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THE UNIVERSITY OF ALBERTA

Production of inhibitory substances by lactic acid bacteria in ground beef

by

© Renate P. Schoebitz

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

FOOD MICRIOBIOLOGY

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

Fall, 1988

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Michael G. Stiles

.....

Supervisor

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.....

.....

Date July 29, 1988

Dedicated to

Luigi, Carlos Alberto, Andres and Roberto

ABSTRACT

Developments in the packaging of fresh meats have created the potential for greater control over the microbial flora on wholesale and (or) retail meat cuts. Attempts to control the contaminating (adventitious) microflora of ground beef by adding lactic acid bacteria has not proven entirely successful. The initial objective of this study was to determine the effect of selected lactic acid bacteria of meat origin have on "contaminating" microflora consisting of *Staphylococcus aureus, Pseudomonas fluorescens, Escherichia coli* and *Klebsiella pneumoniae*. The added lactic acid bacteria consisted of an apparently nonbacteriocinogenic strain (UAL3) and a possible bacteriocinogenic strain (UAL59). Both strains showed an inhibitory effect on the contaminating microflora, especially when they were added at a concentration of 10^6 CFU/g. This was in part a pH effect, but in certain circumstances, neither pH, nor lactate, nor H_2O_2 production could explain the inhibitory effect, and the possibility of a bacteriocin-like inhibitory substance causing the effect was apparent. Further studies involved the ability of a bacteriocinogenic strain UAL8 to produce its inhibitory substance in meat.

Strain UAL8 is a nonaciduric strain which grows in meat at pH 5.5 but does not produce its bacteriocin-like substance at pH 5.5 or below. It was demonstrated that UAL8 produces its inhibitory substance in artificial growth media at pH 6.0 and during incubation at 4 and 1°C. When UAL8 was inoculated into pH adjusted ground beef, initial experiments failed to demonstrate the production of the inhibitory substance in the meat extract. Modification in the extraction procedures to eliminate coprecipitation of the inhibitory substance with the meat proteins, use of γ-irradiation to inactivate the producer strain UAL8, modification of the storage temperature to reduce protease activity and a change in type of membrane filter resulted ultimately in the demonstration of active bacteriocin-like inhibitory substance(s) in the meat. This indicates that production of bacteriocin like inhibitory substances by the lactic acid cultures in meat is a property worthy

of consideration in developing suitable starter cultures intended to predominate the bacterial population of anaerobically packaged meats.

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1.INTRODUCTION

The potential for controlling the microbial flora of meat has increased with the packaging of fresh and processed meats in plastic film. After packaging in an hermetically sealed plastic bag, changes in the microbial flora depend on the microbial load of the meat at packaging, the gas permeability of the plastic wrap, the gas atmosphere in the bag, time and temperature of storage. It has been well established that under anaerobic conditions caused by vacuum or modified gas atmosphere (MA) packaging of meat, the typical aerobic spoilage microflora of meats is inhibited, while a facultative anaerobic microflora consisting mainly of lactic acid bacteria (LAB) is able to grow and predominate the meat microflora (Christopher *et al.*, 1980; Lee *et al.*, 1984, 1985). This has had a dramatic effect on the shelflife of fresh and processed meats. At storage temperatures below 4°C and preferably at -1°C, storage life of anaerobically packaged fresh meat has been extended to 4-6 weeks (Blickstad *et al.*, 1981; Gill and Penney, 1985). Under these conditions, spoilage of meat is usually caused by souring or off odors produced by the LAB microflora. In general, attempts to extend the shelflife of anaerobically packaged meats by the addition of a lactic microflora have not been successful. In the 1970's, the first attempts were made to use LAB as biologic systems for the extension of shelflife by inhibiting the spoilage flora of aerobically stored meats. Later the same principle of LAB as starter cultures was applied to vacuum packaged (VP) meats (Reddy *et al.*, 1970, 1975; Daly *et al.*, 1970). The inoculated LAB flora was changed from strains of dairy origin, as used by Reddy *et al.* (1970) to meat isolates (Hanna *et al.*, 1980, 1983; Schillinger and Luecke, 1986) but always using large numbers of LAB as the starting inoculum to get the desired inhibitory effect on the "contaminating" microflora (Raccach and Baker, 1978; Raccach *et al.*, 1979).

LAB produce a variety of antagonistic substances such as hydrogen peroxide, metabolic end products such as lactic acid, antibiotic-like substances and bactericidal proteins termed bacteriocins (Klaenhammer, 1988). In recent years, the production and

characterization of bacteriocins produced by LAB has received increased attention (Zajdel *et al.*, 1985; Joerger and Klaenhammer, 1986; Hoover *et al.*, 1988). The inhibitory spectrum of some bacteriocins such as Lactocin 27 (Upreti and Hindsdill, 1973) and Lactacin B (Barefoot and Klaenhammer 1984) is narrow, being effective against closely related species. Some gram positive microorganisms, however, produce bacteriocins that are active against a wider spectrum of bacteria, for example bacteriocins produced by *Pediococcus acidilactici* and *Pediococcus pentosaceus* (Hoover et al., 1988).

Several characteristics are desirable for LAB strains to be considered for use in the extension of shelflife of VP meats. The strains must be able to grow well at refrigeration temperatures and they must be able to outnumber the adventitious LAB present in meats. If dominance of the microflora depends on the production of bacteriocins or bacteriocin-like compounds, they must be able to produce these compounds at low temperature, and the LAB must grow and produce bacteriocin at the pH of meat, i.e. 5.5 or lower. Another requirement is that the LAB strains should not cause excessive souring of the meat due to the production of lactic acid. The bacteriocin producing capacity is often plasmid mediated and through techniques of genetic engineering it might be possible to combine these attributes and "produce" the ideal starter strain for fresh meats. The use of a combination of LAB strains as used in the dairy industry may also represent a possibility.

The objective of this study was to determine the inhibitory activity of two selected LAB strains on bacterial spoilage microflora and potential pathogens that might contaminate VP ground beef. In the second part of the study, the production of an inhibitory substance by a selected LAB producer strain was tested in VP ground beef at refrigeration temperature.

2.LITERATURE REVIEW

2.1 Bacterial spoilage of meat

Bacterial spoilage of meat may be defined as the state where changes due to growth or metabolic activity make the meat unacceptable for human consumption as a result of off odor, color or flavor (Gardner, 1983). Deep tissue of an healthy animal is sterile at time of slaughter. Bacterial growth in meat is mainly due to surface contamination of the carcass from hides, feet, equipment and handling (Nottingham, 1982; Dainty *et al.*, 1983).

Bacteria do not penetrate into muscle tissue until high cell numbers are reached and spoilage has developed (Gill and Penney, 1977; Gill, 1986; Kraft, 1986). Bacterial penetration into the tissue is a slow process since proteolytic enzymes are only produced in the late log phase of growth (Gill and Penney, 1977). Among the enzymes naturally present in meat are proteases, but their role in post mortem muscle degradation appears limited. These meat proteases are divided into neutral proteases which are active around pH 7.0, and cathepsins which become active at pH 5.0 to 6.0 (Greaser, 1986).

The type of bacterial flora predominating in meats varies with conditions of meat storage. In aerobically stored meats, gram negative aerobic nonsporeforming bacteria predominate. *Pseudomonas* is the main bacterial genus present, followed in lower numbers by the genera *Acinetobacter*, *Moraxella* and *Flavobacterium* (Jay and Shelef, 1978). In anaerobically (vacuum packaged or modified atmosphere) stored meats, gram positive bacteria predominate, particularly lactic acid bacteria (LAB) and *Brochothrix thermosphacta* (Sutherland *et al.*, 1975; Christopher *et al.*, 1979). In cured meat products, gram positive bacteria also form the largest group of bacteria and high salt concentration favors the growth of fungi (Kraft, 1986).

When microorganisms grow in meat they utilize low molecular weight compounds such as glucose, glucose-6-phosphate, amino acids and lactic acid (Farber and Idziak, 1982). *Pseudomonas* spp. in aerobically stored meats preferentially utilizes glucose.

During this process no compounds causing off odors are formed. When the glucose is depleted, amino acids are attacked and sulfides, esters and acids are formed (Gill, 1976, 1986). At concentrations of *Pseudomonas* of 10^6 CFU/cm^2 off odors are not detected, whereas at 10^7 CFU/cm^2 detectable spoilage begins, principally due to the development of off odors. At 10^9 CFU/cm^2 slime accumulates at the surface of meat (Egan, 1984).

Dark firm and dry (DFD) meat has a final pH above 6.0. This phenomenon is seen more frequently in bull meat and is due to the depletion of glycogen by stressed animals before slaughter. The absence of glycogen reduces lactic acid formation and hence the normal drop in pH does not occur. DFD meat cannot be stored under VP conditions because the high pH favors growth of *Alteromonas putrefaciens*, a strongly proteolytic microorganism, which causes spoilage by "greening" of meat due to break down of sulfur containing amino acids (Newton and Gill, 1978; Gill, 1986).

2.1.1 Tests for determination of meat spoilage

Meat spoilage is usually determined by the number of aerobic nonsporeforming bacteria present or by evaluating the organoleptic characteristics. Alternative tests are the chemical changes that take place during bacterial growth such as ammonia production, extract release volume and pH (Egan, 1984). Nassos *et al.* (1983, 1985) studied the possibility of predicting early spoilage of anaerobically stored meat by measuring the amount of lactic acid produced. A direct correlation was found between drop in pH, lactic acid production and the presence of gram positive bacteria in the meat.

Recently a different approach for predicting meat spoilage has been used. This involves the detection of diamines such as putrescine and cadaverine in VP beef (Edwards *et al.*, 1985). It was found that after 7-8 weeks of anaerobic storage at 1^{o}C the diamine concentration correlated better with the presence of LAB than with gram negative bacteria. Cadaverine concentration increased more rapidly than putrescine. Measurable amounts of diamines were detected before off odors could be detected organoleptically. Total viable

bacterial count is not of great use for determination of spoilage in VP meats. The presence of diamines is also not evidence for spoilage, but they can serve as a guide for shelflife of the product.

Edwards and Dainty (1987) studied the volatile compounds associated with spoilage in the head space of VP pork stored at 5°C. A comparison of head space gases of normal and high pH pork showed that the main difference was the presence of sulfur containing compounds in high pH pork. Of these gases, methanethiol, a metabolite from methionine, was the main gas present. A possible source is the break down products from the metabolic activity of *A. putrefaciens* or *Enterobacteriaceae*.

2.1.2 Methods for control of meat spoilage

Different methods for control of spoilage and sanitizing of carcasses for the extension of shelflife of fresh meat have been studied. These include the addition of glucose, reduction of pH and treatment with organic acid . Spraying with organic acids has proven successful in reducing microbial flora on carcasses, but problems with discoloration could not be overcome (Cacciarelli *et al*., 1983), except when a concentration of 1.25% lactic acid was used (Smulders and Woolthuis, 1985). The use of 2 to 3% sodium lactate has been claimed to be successful in extending the shelflife of meats and with preservation of the meat color (Duxbury, 1988). Automated pressure spray washing of carcasses with water also proved effective in reducing counts of *Enterobacteriaceae* (Crouse *et al*., 1988).

Other methods for the control of meat spoilage are related to storage conditions such as low temperature, modified atmosphere or vacuum packaging and the addition of LAB cultures for the production of bacteriocins such as nisin (Wang *et al*., 1986).

2.2 Lactic acid bacteria in meat

2.2.1 Evolutionary changes of LAB

The term LAB comprises a large group of microorganisms including groups or species of *Streptococcus, Pediococcus, Leuconostoc, Carnobacterium, Lactobacillus* and *Bifidobacterium*. The common characteristics of this group of bacteria are that they are gram positive, nonsporeforming, microaerophilic and their main fermentation product from carbohydrates is lactate (Kandler, 1983). With the exception of *Bifidobacterium*, all genera of LAB are phylogenetically related and according to Fox *et al.*, (1980) they have evolved from a common clostridial type ancestor. The bifidobacteria evolved from an actinomyces branch with the propionibacteria (Fox *et al.*, 1980). The separate grouping of LAB and bifidobacteria parallels their different ecological niches. While LAB are members of the indigenous flora of raw food and are of direct use in the food industry, *Bifidobacterium* spp. colonize the intestine of man and animals and are found in substrates contaminated with feces (Mundt and Hammer, 1968; Kandler, 1983).

The evolution of the LAB from a clostridial type ancestor caused them to develop defenses against oxygen toxicity. While clostridia adapted to an environment without oxygen, other cells developed enzymatic defenses such as production of superoxide dismutase for protection against toxic forms of oxygen (Daeschel *et al.*, 1987). Another defense mechanism used by some cells is the intracellular accumulation of Mn^{2++} which affords the bacterial cell the following mechanism for protection against oxygen toxicity:

$$O^{2-\cdot} + 2H + Mn^{2++} \rightarrow H_2O_2 + Mn^{2++}$$

This mechanism has been observed in certain species of LAB, in particular those associated with plants such as *Lactobacillus plantarum, Lactobacillus fermentum, Pediococcus pentosaceous* and *Leuconostoc mesenteroides*, while those of dairy and meat origin have the superoxide dismutase mechanism. Plants are high in Mn^{2+} content compared with milk or meat, which could explain the different mechanisms acquired (Archibald and Fridovich, 1981).

2.2.2 Classification of LAB from meat

Fermentation of glucose by LAB can be of 2 types: homolactic in which lactic acid is the only end product, and heterolactic in which the formation of lactic acid, acetic acid or ethanol occurs (Kandler, 1983). The classification of LAB isolated from VP meats has not advanced to the same extent as other foods. Until a few years ago a broad group of unidentifiable LAB was grouped into homofermenters or "unclassified streptobacteria", heterofermenters and motile lactobacilli (Hitchener *et al.*,1982). Shaw and Harding (1984) determined the existence of 3 clusters of LAB isolated from VP beef, pork, lamb, and bacon. Cluster I was described as homofermentative and nonaciduric because of their inability to initiate growth at pH 3.9; Cluster II was also homofermentative but aciduric, being able to initiate growth at pH 3.9. A high salt tolerance was also described for LAB from cluster II. Cluster III was the smallest group including only heterofermentative strains, such as *Leuconostoc*. spp.

2.3 Modified atmosphere and vacuum packaging of meats

In recent years, methods for beef distribution have changed from shipment of carcasses as "hanging" quarters or sides to shipment of primal or subprimal cuts (2-9 Kg) as "boxed beef". These smaller sized units are packaged under vacuum in bags with low gas permeability (Seideman and Durland, 1983, 1984). VP provides a method of prolonging the shelflife and palatability of fresh beef during extended periods of shipment and storage. Additional advantages include reduced weight loss, increased hygienic control, controlled aging and color preservation by the formation of reduced myoglobin due to exclusion of oxygen. Disadvantages include cost of the impermeable film, loss due to purge, moisture loss and temperature abuse during shipping and handling which permits development of anaerobic bacteria at ambient temperature and constitutes a potential health hazard (Seideman and Durland, 1983; Genigeorgis, 1985). The physical properties of the impermeable film used in VP are one of the most important factors in maintaining meat

quality. Loss of vacuum and entry of oxygen into the packages results in an environment more deleterious to meat than storage in air, since humidity is high in the vacuum package. Temperature abuse by packers and retailers also occurs with these products since the superiority of these meats compared with fresh meat is well known (Seideman and Durland, 1983).

2.3.1 Historical aspects of modified atmosphere storage of meats

During the 1930's, CO_2 was added to the air in ship lockers to extend the storage life of chilled beef transported to Britain from New Zealand and Australia (Newton ., 1977). In the 1950's, the bacterial flora of fresh, raw meats was extensively studied. Kirsh *et al.* (1952) studied the bacteriology of aerobically packaged refrigerated ground beef and stated that the majority of bacteria isolated were nonpigmented *Pseudomonas* or *Achromobacter* spp, but lactobacilli were also mentioned. Ayres (1960) found that the pseudomonads were responsible for slime production on beef kept at 10°C or lower. Halleck *et al.* (1958) reported that two groups of bacteria dominated the flora of meats packaged in oxygen permeable films. During the first two weeks *Lactobacillus* spp. and nonpigmented aerobic gram negative spoilage bacteria were present, while later, *Pseudomonas fluorescens* became the dominant bacterium. Jaye *et al.* (1962), using films with different gas permeabilities and storage temperatures of -1 and 3°C, concluded that meat stored at low temperature in oxygen impermeable film retained its acceptability for longer periods of time than aerobically packaged meats.

Ingram (1962) reviewed the microbiological principles in prepackaging of meats to illustrate some of the concepts and to lessen confusion about the effects of anaerobic meat packaging. Many of the principles that he discussed remain valid today. In the 1960's, packaging techniques were primarily aimed at preservation of the bright red (oxygenated) color of meat. This was done by packaging in oxygen permeable films. This leads to rapid spoilage due to growth of the aerobic spoilage flora. The concentration of oxygen present

in a pack influences the microbial flora on the meat (Ingram, 1962). Oxygen in the package is consumed by the meat and the microorganisms. At the same time carbon dioxide is released. It is the partial pressure of these gases in the bag that influences the microbiological flora that develops. This in turn depends on the gas permeability of the wrap. A balance is established between the metabolic exchange rate of the contents and the diffusion rate through the wrap.

2.3.2 Effect of different gas atmospheres on meat storage

Considerable effort has been directed toward finding the ideal internal atmosphere and wrap that will preserve meat color and quality over an extended period of time. Different gas combinations of CO_2, N_2, O_2, air or VP have been tested on different meat types. Hess *et al.* (1980) studied the effects of packaging in MA containing air, CO_2, and mixtures of CO_2 and N_2, on the shelflife of beef steaks, veal, pork chops and chicken stored at 1°C. They found that shelflife was generally increased when modified atmospheres were used and that the best results were obtained with 100% CO_2. No comparison was made with VP meats. Huffman *et al.* (1975) also reported that 100% CO_2 was the most effective MA for controlling bacterial growth on beef steaks stored at -1°C for 27 days compared with N_2, O_2, air or a gas mixture. Lee *et al.* (1983, 1984, 1985) reported similar bacterial counts between VP and N_2 packaging in studies on veal, beef and pork during 49 days storage at temperatures below 0°C. Similar increases in bacterial numbers between treatments were observed as storage temperatures were increased, regardless of the MA used.

In studies on the microbial quality of pork and beef stored in different gas atmospheres at -1°C, Huffman and co-workers (1974, 1975) reported good results for CO_2 and a mixture of gases containing CO_2, while no difference in counts was observed between storage in air, O_2 or N_2. According to Seideman and Durland (1983), results for the use of N_2 have been contradictory. This gas only replaces O_2, it does not have the

same inhibitory effect as CO_2. They also stated that bacterial counts for VP meat were intermediate between O_2 and CO_2 atmospheres. N_2 is often used as a "filler gas" in combination with CO_2. When the latter is absorbed into the meat during storage, N_2 prevents the bag from collapsing onto the meat surface. The use of combinations of gases such as CO_2 and N_2 or CO_2, N_2 and O_2 have not given clear cut results and different effects have been reported by various workers. The most effective gas has always been CO_2, especially when used alone (Huffman, 1974; Huffman *et al.*, 1975; Newton *et al.*, 1977; Erichsen and Molin, 1981; Christopher *et al.*, 1980).

2.3.3 Mode of action of CO_2

The inhibitory effect of CO_2 on the microbial flora of meat was recognized in the 1930's (Haines, 1933) when it was shown that inhibition was not only due to a pH effect but also some other unknown mechanism. Increased inhibition of the aerobic gram negative spoilage microflora occurs as the incubation temperature is lowered (Coyne, 1933). The exact mode of action of CO_2 on microbial growth has still to be clarified. It is known that CO_2 extends the lag phase and generation time of microorganisms. Increased inhibition is seen at refrigeration than at higher temperatures due to the increased solubility of the gas in the water phase at lower temperatures (Blickstad and Molin 1983b; Daniels *et al.*, 1985). Studies of cellular interaction with carbon dioxide have indicated that CO_2 interferes with various chemical processes of metabolism and that it alters membrane fluidity affecting permeability and transport Also a specific effect of CO_2 on certain metabolic enzymes such as isocitrate- and malate dehydrogenase has been shown (King and Nagel, 1967; Enfors and Molin, 1978).

Ingram (1962) noted that the inhibitory action of CO_2 is selective, a fact that is likely to influence the nature of the organisms that survive. In early studies of meat processing using CO_2, it was noticed that the typical spoilage microflora capable of growing at low temperatures was inhibited (Haines, 1937). It is now generally agreed that

the accumulation of CO_2 in VP meats or the addition of CO_2 selectively inhibits the gram negative spoilage bacteria, in particular pseudomonads, while it allows the growth of lactic acid bacteria (King and Nagel, 1975; Dainty *et al.*, 1979; Blickstad *et al.*, 1981).

2.3.4 Bacteriological aspects of modified atmosphere storage of meats

Lactic acid bacteria represent a low proportion of the initial microbial population on VP meat, but after 20-30 days storage at refrigeration temperature they constitute 70-90% of the total bacterial population (Enfors *et al.*, 1979; Christopher *et al.*, 1980; Egan and Shay, 1982). On the other hand, the aerobic gram negative spoilage bacteria, in particular pseudomonads, are inhibited by the accumulation of CO_2, which can reach concentrations of 20-60% in the bags (Blickstad and Molin, 1983a). Bacterial antagonism between aerobic spoilage bacteria and LAB is another possible cause of this inhibition. Collins-Thompson and Rodriguez Lopez (1980) observed that *B. thermosphacta* declined in numbers when LAB reached 10^6 CFU/g of VP bologna. Similar inhibitory effects were noticed by Dainty *et al.* (1979) for bacterial growth on VP beef. After three weeks storage the level of *B. thermosphacta* and gram negative bacteria had declined, while LAB were still increasing in numbers.

The maximum "aerobic total count" of bacteria detected in VP meat is about one tenth of the maximum found with aerobic storage in a gas-permeable film (Roth and Clark, 1972). This was previously noted by Ingram (1962) in his review, in which he stated that, in a gas permeable film spoilage follows the course of unwrapped meat, while at low O_2 concentration the number of organisms able to grow under anaerobic conditions increases. Initial numbers and types of microorganisms present in meat before storage under MA are important. According to Foegeding *et al.* (1983), high initial numbers of aerobic spoilage bacteria lead to a shorter shelflife of the product, since LAB are unable to outgrow the spoilage microflora. High levels of CO_2 (100%) permit the total domination of LAB. High concentrations of CO_2 also reduce the growth rate of these bacteria. As a

consequence, the shelflife of meat stored at elevated levels of CO_2 can be extended beyond that of VP meats. This was shown by Blickstad *et al.* (1981) and Blickstad and Molin (1983b), who stored pork for about three months at low refrigeration temperature without detectable spoilage.

Another important consideration in vacuum or CO_2 packaged meat is the residual effect of the gas on the microflora when it is subsequently wrapped in gas permeable film. Madden and Moss (1987) demonstrated a lag phase of 3 days in the growth of the aerobic bacterial flora after storage of the meat for 2 weeks in 20% CO_2. After 4 weeks of storage under the same conditions and subsequent aerobic display, a slight increase in aerobic bacterial counts was seen after 7 days. A similar effect was reported by Enfors *et al.* (1979) who stored pork for 21 days at 4°C under different MA conditions with subsequent aerobic display at 4°C for 3 days. Samples stored for 7 days in CO_2 showed a more rapid increase in total aerobic counts when exposed to air compared with those stored for 21 days in CO_2.

B. thermosphacta is a spoilage bacterium that is frequently isolated from VP meats, in which it can achieve levels of 10^6 CFU/g. According to Campbell *et al.* (1979), this organism requires a pH of 5.8 or higher and a minimal amount of O_2 for growth. It was first reported in high numbers in VP lamb (Barlow and Kitchell, 1966). Information about its growth on VP beef and pork is inconsistent (Roth and Clark, 1972; Erichsen and Molin, 1981). According to Gill and Penney (1985), adipose tissue has a pH value close to neutrality because there is no significant respiratory activity, this results in a heterogeneus environment for microbial growth and could explain the different findings between meat types.

2.3.5 Organoleptic properties

Color plays an important role in consumer acceptance of beef. VP beef must be able to "bloom" when exposed to air by forming oxymyoglobin. The color of meat is

greatly influenced by the gas atmosphere in the meat pack. Reduced myoglobin is purple and is the predominant form that occurs in the absence of oxygen, while metmyoglobin is brown in color and is formed in the presence of 1% or less of oxygen (Seideman and Durland, 1984). The addition of gases to the pack has different effects on meat color. Seideman *et al.* (1979), in a study on beef steaks, observed that 100% CO_2 caused surface discoloration while steaks packed in 100% N_2 were similar in rating to VP steaks, illustrating the minimal effect that N_2 has on meat color. Huffman *et al.* (1975) on the other hand found that samples stored for 23 days in 100% N_2 had the least desirable color compared with storage in air, O_2, CO_2 or a gas mixture.

Bacterial growth on the meat surface also influences the color of meat. The high O_2 demand of the aerobic spoilage microflora leads to rapid formation of metmyoglobin. Facultative anaerobes such as LAB do not cause this problem. Some microorganisms also produce hydrogen sulfide, which causes green discoloration of VP meats (Seideman *et al.*, 1984; Cross *et al.*, 1986). Another color defect found mainly in beef is the dark, firm and dry meat (DFD), characterized by a high pH (above 6.0), which is caused by stress conditions before death and causes antemorten depletion of glycogen. The meat appears dark due to an increase in the water holding capacity and consequent increase in light reflection (Gill, 1986).

Ingram (1962) reported that aerobically stored meats spoil with development of putrid odors due to amino acid breakdown, while in the absence of oxygen an acid (sour) spoilage occurs. The latter is caused as a result of lactic acid produced and gives VP meat a characteristic cheesy odor (Jaye *et al.*, 1962). According to some authors this odor disappears soon after opening the meat bag (Schillinger and Luecke, 1986). In DFD meat, offensive, putrid and sulfur-like odors are produced, mainly due to the growth of *Enterobacteriaceae* and *Alteromonas putrefaciens*, which outgrow the LAB (Edwards and Dainty, 1987).

2.4 Meat fermentations

Lactic acid producing starter cultures used in meat fermentations may be fresh, frozen or freeze-dried cultures of the microorganism. They can be single or mixed strains with certain metabolic (enzymatic) properties which are utilized during defined processes in the food industry (Coretti, 1977). The original starter bacteria for use in dairy products were isolated from sour milk, but their natural ecological sources are soil, plant surfaces and the rumen of bovine animals. Their use in the fermentation of food and beverages gives the product certain characteristic organoleptic properties (Lewis, 1987).

Among the undesirable substances that may be produced by LAB in food is carbon dioxide, which is produced by heterofermentative species. During the fermentation of cucumbers by *Leuconostoc mesenteroides*, this gas causes the formation of hollow centers in the vegetable, a phenomenon also known as "cucumber bloating". In sauerkraut fermentation, *L. mesenteroides* can cause the defect known as "slimy" or "ropy" kraut, due to the accumulation of dextrans that give sauerkraut a slimy characteristic (Daeschel *et al.*, 1987). Undesirable characteristics of fermented meat products can be caused by LAB. *Lactobacillus viridescens* is a heterofermentative organism that causes major spoilage losses of meats due to greening and gas formation, producing color defects as well as swelling of canned and vacuum packaged meats (Tompkin, 1986). Excess acid production occurs if fermentation time is prolonged.

2.4.1 Starter cultures in the processed meat industry

In the meat industry, manufacturers traditionally depended upon the development of the naturally occurring microflora to get the desired end product. Another technique in the production of processed meats is the inoculation of a portion of a successful, recently fermented meat into a freshly prepared batch, a technique known as "back-slopping" (Bacus and Brown, 1981). In recent years, however, more meat processors have started using specific starter cultures to assure more rapid and controlled predominance of the

desired microorganism. This has the advantage of shortening the ripening time from several days to 6-7 hours and standardizing the product (Luecke, 1985; Bacus, 1986).

Initial studies on the development of starter cultures for the meat industry were done in the 1940's, after the successful introduction of starter cultures in the dairy industry. The original strains used for meats were from dairy sources, but the dairy strains did not proliferate in the meat environment, probably because of a lack of tolerance to salt and (or) nitrites (Bacus and Brown, 1981). In the 1960's, the first commercial starter culture for processed meats became available in lyophilized form, under the trade name of "ACCEL" and consisted of a strain of *Pediococcus acidilactici* (Deibel *et al*., 1961). Later a frozen concentrate of the culture was introduced to avoid the rehydration period, to achieve more consistent results and to shorten the lag phase (Bacus, 1986).

The primary functions of the starter culture in fermented meat products are to produce lactic acid from glucose, to lower the pH and to inhibit growth of other bacteria. Most isolates from naturally fermented meats belong to the genus *Lactobacillus*. These generally grow at lower temperatures than *P. acidilactici*, which makes them more suitable in the manufacture of dry sausages (Bacus, 1986). Some processed meats such as dry sausages are not subjected to a significant degree of heat treatment during manufacture. The safety of these products depends on salting, curing, fermentation and drying. Most pathogenic bacteria are inhibited by the addition of salt and nitrite, allowing the proliferation of LAB, yeasts and other gram positive species (Bacus, 1984). The use of starter cultures in processed meats has not only the advantage of shortening the fermentation time and assuring a product of consistent quality, but it also ensures the product's safety against the growth and survival of pathogenic microorganisms such as *Staphylococcus aureus* and *Salmonella* spp. (Bacus, 1986).

S. aureus intoxication from the consumption of sausage is not uncommon in North America (C.D.C, 1979; Health and Welfare Canada, 1986), and it is often caused by growth of *S. aureus* in a defective fermented dry or semidry sausage. *S. aureus* generally

multiplies and produces toxin during the initial stage of sausage fermentation, growing only in the outer portion of the product. A low final pH, low water activity or high salt concentration does not prevent toxin formation if the bacterium had a chance to reach counts of 10^6 CFU/g (Bergdoll, 1980; Bacus and Brown, 1981). This pathogen however is a poor competitor. The advantage of using starter cultures is the rapid production of acidity and development of high numbers of LAB within a short time, which supresses the growth of *S. aureus* . Inhibition can also occur due to the production of peroxides or other substances. In raw meats this pathogen is repressed by the natural microflora (Smith and Palumbo 1981; Luecke, 1985).

Salmonella is also a potential risk in processed meats because it may be present in high numbers in raw meats, particularly pork and poultry. Certain factors, such as high initial pH and water activity, low amount of fermentable carbohydrate and a low number of lactobacilli in the raw sausage mixture, favors the growth of *Salmonella* in these products (Bacus and Brown 1981; Luecke, 1985). Salmonellosis is a common problem associated with the consumption of fresh spreadable sausage meats in Germany. To retain the fresh taste of the products, they are manufactured with little or no added sugar or acidulants, which permits the growth of the pathogen and other *Enterobacteriaceae* (Luecke, 1985). In the United States, however, this type of sausage is not popular, and *Salmonella* are rarely implicated in food-borne illness due to consumption of fermented meats. Experimental work has indicated that lactic acid is an effective agent against *Salmonella* (Bacus, 1986). The degree of inhibition is dependent upon the species and strain of LAB, amount of LAB starter inoculum, load of pathogenic microorganisms, incubation temperature and other factors (Park and Marth, 1972; Sirvioe *et al.*, 1977; Raccach and Baker, 1978; Raccach *et al.*, 1979).

Other bacterial pathogens that could be present in fermented meats, particularly dry sausages, are *Clostridium botulinum* and *Clostridium perfringens*.. Their occurrence is rare because of their poor ability to compete with other bacteria (Bacus, 1984). According

to Luecke (1985), there is no evidence that *C. botulinum* will grow and form toxin during ripening and storage of correctly fermented sausages. *C. botulinum* is controlled by low pH and low water activity, independent of the addition of nitrite. In a study by Gibbs, (1987) botulinum toxin was only detected in sausages when glucose was not added and the pH did not fall below pH 5.5. A new development in bacon production is the so-called "Wisconsin Process" in which, by the addition of a starter culture, the amount of nitrite is lowered but the necessary protection against botulinal toxin production is achieved. For this process, a strain of *P. acidilactici* and sucrose are added to the curing brine. The starter culture grows slowly at 7°C, but rapidly at 23-27°C, producing acid from sucrose if temperature abuse occurs (Tanaka *et al.*, 1985).

Yersinia enterocolitica is another pathogen that has been isolated from meats in recent years, particularly fresh pork. Since pork is a major ingredient in sausage formulations and some sausages are not heat treated, the presence of LAB, curing salts and spices are important for control of the growth of *Y. enterocolitica*. Raccach and Henningsen (1984) tested the inhibitory effect of three LAB strains and found good inhibition at two concentrations of the starter culture against two serotypes of *Yersinia*. They concluded that the presence of lactate, low pH and curing salts had an inhibitory effect.

2.4.2 Starter cultures for the extension of shelflife of fresh or vacuum packaged meats

Storage of meat under aerobic conditions allows rapid spoilage due to growth of gram negative nonsporeforming bacteria. When meat is stored under anaerobic conditions, LAB constitute the main microflora. Their ability to grow at low pH, increased levels of CO_2 and under anaerobic conditions allows them to reach high numbers in vacuum packaged meats (Egan, 1983).

The use of LAB cultures in the fermented meat industry has gained wide spread acceptance. Their application for the extension of shelflife in fresh meats, however, has not been developed. Reddy *et al.* (1970) conducted a study in which LAB were used to extend the shelflife of aerobically stored ground beef. The meat was inoculated with *Streptococcus lactis, Leuconostoc citrovorum* or a mixture of both organisms of dairy origin. The results showed good inhibition of the gram negative flora in aerobically stored meat over 7 days. The inhibitory effect increased with increased inoculum size. The addition of ascorbic acid improved or retained the color compared with addition of LAB alone. A similar result was observed with the inoculation of beef steaks (Reddy *et al.*, 1975). Gilliland and Speck (1975), using different LAB strains, also observed inhibition of the psychrotrophic spoilage microflora and concluded that at least part of this effect was due to production of hydrogen peroxide.

Roth and Clark (1975) reported that the growth of *B. thermosphacta* was inhibited by *Lactobacillus* spp. in vacuum packaged meat. They stated that the inhibition was not due to CO_2 or acid and that this bacterium started growing after vacuum packaging, but stopped growth once the LAB reached their maximum numbers. This effect was also observed in vacuum packaged bologna, particularly with strains of *Lactobacillus brevis* and *Lactobacillus plantarum*, which had been isolated from the same sausage. The antagonistic effect was not influenced by temperature, since it was observed at 5 and 15°C (Collins-Thompson and Rodriguez Lopez, 1980). It was also shown that the inhibition of *B. thermosphacta* was not due to acidity, lactic acid or hydrogen peroxide and that some specific antibiotic substance produced by the LAB caused the effect (Collins-Thompson and Rodriguez Lopez, 1982).

Raccach and Baker (1978) and Raccach *et al.* (1979) showed that *P. cerevisiae* and *L. plantarum* inhibited the growth of *S. aureus* and *Salmonella typhimurium* when inoculated into cooked poultry meat or broth at a concentration of 10^9 CFU/g, without causing a drop in the pH of the meat. A greater effect was noticed in broth than in meat.

Recently, a great amount of work has been done on the effect of certain lactic starter cultures on spoilage and pathogenic microorganisms in meat (Dubois *et al.*, 1979; Hanna *et al.*, 1980; Abdel-Bar and Harris, 1984; Schillinger and Luecke, 1986). Many difficulties must be overcome before LAB can be used commercially as starter cultures for the extension of shelflife of meats. In most studies large inocula were necessary to achieve the desired effect. From a practical viewpoint, this would not only increase the cost of the product, but it would also cause undesirable organoleptic changes during storage, affecting the color and odor of meat. Smith *et al.* (1980) showed that beef steaks inoculated with *Lactobacillus* species had a higher incidence of off odors, surface discoloration and poor flavor ratings compared with uninoculated control samples. The authors concluded that the disadvantages outweighed the benefits obtained from the inoculation of meat with LAB. Future work should be directed to looking for favorable traits in different LAB strains and, through selection or genetic engineering, to search for starter strains that combine the desired characteristics for a starter culture for use in fresh, vacuum packaged meat.

2.5 Bacteriocins

The term bacteriocin refers to antimicrobial substances of protein nature, that are produced by bacteria which demonstrate activity against other bacteria of related or unrelated species (Tagg *et al.*, 1976).

2.5.1 Historical perspective

The study of antagonistic interactions between bacteria dates back to the 1800's when Pasteur and Joubert (1877) noted the inhibitory effect of some bacteria on *Bacillus anthracis*. The main concern in those early days was to find a method of controlling diseases such as anthrax and diphtheria by using nonpathogenic, antagonistic microorganisms, rather than studying the chemical nature of the inhibitory substances that are produced. Although the inhibitory substances were not identified, according to Tagg *et*

al. (1976), it seems likely that many of the interactions observed were caused by substances now classified as bacteriocins. The study of bacteriocins dates back to 1925 when Gratia published his observations on an antibacterial substance, now known as colicin. His observations were made on a strain of *Escherichia coli* which produced a substance bactericidal to another strain of *E. coli*. Later, Fredericq (1946, 1957) studied and classified the colicins and also reported on the methods used to demonstrate them. Colicins are produced mainly by *E. coli* and *Shigella* spp., and they were an on-going source of research interest during the 1950's and 60's. Gardner (1949) reported on an antibiotic-like substance produced by a strain of *S. aureus*. Jacob *et al.* (1953) renamed the colicins to include substances produced by other groups of bacteria, and the term "bacteriocins" was introduced.

De Klerk and Coetzee (1961) published the first study on the characterization of bacteriocins produced by gram positive bacteria, specifically strains of *Lactobacillus*. They considered that the antibacterial spectrum was restricted to members of the family *Lactobacteriaceae*, similar to colicin activity which was restricted to the family *Enterobacteriaceae*. During the 1970's more intense work on the bacteriocins produced by gram positive bacteria was reported and Tagg *et al.* (1976) published an extensive review on bacteriocins from these gram positive bacteria.

2.5.2 Definition of the term bacteriocin

Until the 1970's most investigations on bacteriocins centered on the colicins produced by gram negative bacteria (Tagg *et al.*, 1976). The original definition for bacteriocins given by Jacob *et al.* (1953) was based on criteria given for colicins. These included a bactericidal mode of action, a narrow inhibitory spectrum against homologous species and adsorption to specific host cell receptor sites. Few bacteriocins produced by gram positive bacteria fit these criteria, where a wider spectrum of activity against organisms of different species has often been found (Gagliano and Hinsdill, 1970; Visser

et al., 1986). According to Tagg *et al.* (1976), a variety of inhibitory substances produced by bacteria, including metabolic products, enzymes, defective bacteriophages and classical antibiotics have been referred to as "bacteriocins". There has often been overlap in the definition of these substances and a precise explanation for the term bacteriocin is difficult.

Other characteristics such as the presence of an essential biologically active protein moiety and a bactericidal mode of action have been applied to bacteriocins from gram positive as well as gram negative microorganisms (Tagg *et al.*, 1976). The attachment to specific cell receptors and plasmid borne genetic determinants of bacteriocin production require further study, since variations have been found among strains, and data is not available for many strains (Tagg *et al.*, 1976; Joerger and Klaenhammer, 1986). To overcome the problem of defining bacteriocins, Tagg *et al.* (1976) suggested the use of the term "bacteriocin-like substances" for those substances that are not completely defined, and recommended use of the term "bacteriocin" only for those substances that have been well characterized.

2.5.3 Composition and characteristics of bacteriocins from lactic acid bacteria

Bacteriocins are heterogeneous substances, chemically diverse but with the common characteristic of having an active protein moiety. Some have been described as forming a large protein-lipid-carbohydrate complex, which according to some authors can be fragmented into smaller units, while retaining their activity (Jetten *et al.*, 1972; Barefoot and Klaenhammer, 1984). Others found that the entire unit is necessary to retain the activity (de Klerk and Smit, 1967). The amount of each component present in the bacteriocin complex varies with the bacterial species. For strains of *S. aureus* and *Lactobacillus helveticus*, the main component of their bacteriocins is a protein, while lipids constitute the second largest component (Gagliano and Hinsdill, 1970; Upreti and Hinsdill, 1975). Carbohydrates are present in less than 10% of bacteriocins. This differs from

findings for the bacteriocin from *Lactobacillus fermenti* (de Klerk and Smit, 1967) in which carbohydrate forms 53%, protein 23% and lipid 20% of the molecule. According to Upreti and Hinsdill (1975), the bacteriocin Lactocin 27 produced by a strain of *L. helveticus* is first synthesized as a small molecular weight compound, which then conjugates with others to form a larger complex. The association and dissociation of bacteriocin into subunits was shown to be a reversible process (Jetten *et al.*, 1972), during which the biological activity is retained (Gagliano and Hinsdill, 1970; Barefoot and Klaenhammer, 1984). According to Tagg *et al.* (1976), the presence of the monomer or the complex is dependent upon the pH and ionic strength of the medium. De Klerk and Smit (1967), on the other hand, concluded that the activity of bacteriocins is dependent upon the integrity of the lipoprotein-carbohydrate complex.

Bacteriocins treated with proteolytic enzymes, such as pronase or trypsin, are inactivated. This indicates that the active portion of the complex is the protein (de Klerk and Coetzee, 1961; Dajani and Wannamaker, 1969; Upreti and Hinsdill, 1975; Barefoot and Klaenhammer, 1983). In 1967, Bradley reported that there are low and high molecular weight bacteriocins. Low molecular weight bacteriocins are more susceptible to trypsin and less sensitive to heat inactivation, while the high molecular weight bacteriocins could be phage related.

Bacteriocins can be either cell bound, or released into the medium. Gagliano and Hinsdill (1970) showed that staphylococcin 414 is only released into the medium after disruption of the producer cell. On the other hand, Lactocin 27 produced by *L. helveticus* (Upreti and Hinsdill, 1973) is excreted during cell growth, and no increase in activity occurs after the disruption of the cells. The production of bacteriocin in culture media can be pH dependent, as shown for Lactacin B produced by a strain of *L. acidophilus* (Barefoot and Klaenhammer, 1984). When the pH of the medium was below 5.9 no activity was detected, whereas increased activity occurred toward neutrality. A different result was reported for Helveticin J. This bacteriocin was produced in greatest amount at

pH 5.5 and accumulation occurred in the late logarithmic and stationary phases of growth (Joerger and Klaenhammer, 1986).

2.5.4 Antimicrobial spectra

Antimicrobial spectra of bacteriocins are generally limited to the same or closely related species (Fredericq, 1957; Reeves, 1965). This is not the case to the same extent for gram positive bacteria as it is for gram negative bacteria. For example, the bacteriocin produced by a strain of *Streptococcus faecalis* var. *zymogenes* is not only active against closely related strains, but it is also mainly active against pneumococci (Bottone *et al.*, 1971). Staphylococcin 414, a "bacteriocin" produced by *Staphylococcus epidermidis* is active against different gram positive and gram negative bacteria (Gagliano and Hinsdill, 1970). Visser et. al. (1986) showed that a strain of *L. plantarum* inhibited the gram negative plant pathogens *Xanthomonas campestris* and *Erwinia carotovora* when grown in broth culture. In contrast, Lactacin B produced by *L. acidophilus* inhibited other lactic acid bacteria (Upreti and Hinsdill, 1973; 1975; Barefoot and Klaenhammer, 1983, 1984). According to Klaenhammer (1988), some strains of LAB produce two types of bacteriocins, one with bactericidal action against closely related species and, less common, one that is effective against a wide range of gram positive bacteria. This characteristic could give LAB an additional advantage when competing with other microorganisms in foods.

2.5.5 Mode of action of bacteriocins

The lethal action of bacteriocins occurs in two stages. The first corresponds to the adsorption of the bacteriocin to exposed cell envelop receptors. This could be reversible since no physiological damage occurs to the cell with removal of the bacteriocin by trypsin. A few seconds later, the second irreversible process occurs (Plate and Luria, 1972; Tagg *et*

al., 1976; Zajdel *et al.*, 1985). This suggests that during the second phase the bacteriocin penetrates the cell and acts directly on its biochemical target (Tagg *et al.*, 1976).

The adsorption of bacteriocins is a non-specific process, indicating that the specificity for attachment is not entirely dependent upon the presence of particular receptors on a sensitive cell (Upreti and Hinsdill, 1973, 1975; Tagg *et al.*, 1976; Barefoot and Klaenhammer, 1983, 1984) concluded that in the case of Lactacin B the target and means by which the bacteriocin specificity is determined remain unknown. According to Zajdel *et al.* (1985), the bactericidal effect of Las 5, a bacteriocin produced by *Streptococcus cremoris* , is highly dependent on pH of the medium, but the temperature at which the susceptible cells are treated has no influence on the killing effect of this bacteriocin. They also showed that ions such as K^+, Ca^{2+} and Mg^{2+} can have a protective effect on bacteriocin treated cells. They found that Mg^{2+} was the most protective and that the effect increased with higher ion concentrations.

Bacteriocin-like compounds can have a bactericidal or bacteriostatic effect on susceptible cells. According to Tagg *et al.* (1976), an inhibiting substance should not be classified as bactericidal or bacteriostatic until a wide range of indicator strains have been tested. They also suggest that these variable effects might be due to two or more substances present, with different modes of action against bacterial cells. The majority of reports on a variety of bacteriocins deals with their bactericidal rather than their bacteriostatic effects (Dajani and Wannamaker, 1969; Bottone *et al.*, 1971; Barefoot and Klaenhammer, 1983; Zajdel *et al.*, 1985). On the other hand, Lactacin 27 (Upreti and Hinsdill, 1975) showed only bacteriostatic activity against the indicator *L. helveticus* LS 18. The mode of action was mainly a slowing down of protein synthesis, but changes in the sodium-potassium exchange were noticed, which suggests that the bacteriocin acts on the cell membrane. Staphylococcin 414 on the other hand is bactericidal, but cell lysis was not observed for sensitive broth cultures treated with this bacteriocin (Gagliano and Hinsdill, 1970).

The number of bacteriocin molecules necessary to kill or inactivate a sensitive cell has been studied and there is no agreement that a one to one interaction occurs (Reeves, 1965; Tagg *et al.*, 1976). The effectiveness of a bacteriocin also depends on the physiological state of the indicator microorganism. A bacterial cell is more sensitive when it is actively multiplying. This indicates the requirement for an active cellular metabolism to cause the death of the cell (Tagg *et al.*, 1976).

2.5.6 Immunity of producer cells to their own bacteriocins

To ensure the survival of the bacteriocin producer cells some form of protection must be present to avoid a suicidal activity. Tagg *et al.* (1976) stated that the nature of the producer cell immunity is not well understood, and that a specific immune substance could be produced by the cell. Data from earlier work (Davie and Brock, 1966) indicated that *Streptococcus zymogenes* produces a specific inhibitory substance which neutralizes its own bacteriocin. The inhibitory substance is a ribitol teichoic acid, containing glucose, D-alanine, phosphate and ribitol, which is released by the cell during mid and late logarithmic growth. This explains the loss of activity of the lytic agent during the growth of the culture. A similar phenomenon was observed in a strain of *Serratia marcescens*. Foulds and Shemin (1969) observed that when this strain was grown at 30 to 37°C no bacteriocin activity was detected, whereas at 39°C bacteriocin activity was readily observed. The bacteriocin inhibitor lost its activity when heated at 39°C for 1 h. It appears to be a protease. Jetten *et al.* (1972) noted the presence of a small molecular weight inhibitor which was either produced by *S. epidermidis* 1580 or present in the original medium. Barefoot and Klaenhammer (1983) stated that an *L. acidophilus* producer strain showed autoimmunity against its own Lactacin B by lacking or masking the appropriate receptors in their cells.

2.5.7 Screening procedures for detection of bacterial antagonism

When testing bacterial strains for possible bacteriocin production, the screening procedures used are important. These can be divided into direct or deferred antagonism. For the direct test, producer and indicator organisms are grown simultaneously. Inhibition depends upon the release of a diffusible inhibitor during the early stage of growth of the producer strain. For deferred antagonism the test organism is grown first and then killed by exposure to heat or chloroform, before being overlayered with the indicator strain. When using these screening methods a variety of conditions and media should be tested since the optimal conditions for growth of the test strain are not necessarily the best for bacteriocin production. These methods have the inconvenience of not specifically demonstrating bactericidal activity (Tagg *et al.*, 1976).

2.5.8 Antagonism not related to bacteriocin production

The study of bacteriocin production requires the differentiation of true bacteriocin inhibition from substances or phenomena that mimic their action. Several bacterial products can cause inhibition of a sensitive indicator lawn, including bacteriophages, hydrogen peroxide, lactic acid, antibiotic-like substances such as nisin, ammonia, free fatty acids etc. The difference between bacteriophage and bacteriocin is that the first is able to self-replicate within a susceptible organism. This can be shown by the appearance of plaques of inhibition with increasing dilutions spotted onto an indicator lawn. On the other hand, the inhibitory activity of bacteriocins decreases and disappears with increasing dilution (Tagg *et al.*, 1976).

Hydrogen peroxide when accumulated in a culture medium has a toxic effect, unless the microorganism produces enzymes such as catalase or peroxidase which break down the peroxide. Among the LAB, streptococci often produce hydrogen peroxide. This was shown by Malke *et al.* (1974), who studied different serotypes of group A streptococci and found that 78% of them gave a positive peroxide test. No inhibition was seen under

anaerobic conditions, which is also an indication that hydrogen peroxide is the inhibitory substance.

Another important consideration when working with LAB is the inhibitory effect of lactic acid. This was recognized by Tramer (1966) when he studied the inhibitory effect of *L. acidophilus* on a strain of *E. coli*. He noticed that raising the pH of the broth containing the producer strain above pH 4.5 caused the inhibitory effect to disappear.

Among the antibiotic like substances, produced by *S. lactis* nisin is the most thoroughly characterized. It is proteinaceous and bactericidal to gram positive bacteria and prevents outgrowth of *Clostridium* and *Bacillus* spores. In European countries it is used as an antimicrobial substance in food processing (Klaenhammer, 1988). Walstad *et al.*, (1974) looking for bacteriocin production by gonococci, found that inhibition caused by *Neisseria gonorrhoeae* was due to long chain free fatty acids and phospholipids associated with the cell envelop, and not a bacteriocinogenic effect. Rogul and Carr (1972) found that inhibition by *Pseudomonas pseudomallei* was due to production of ammonia.

These studies emphasize the need to rule out the possibility that inhibitory substances other than a bacteriocin or bacteriocin-like compound are responsible for inactivating the indicator organism before drawing conclusions about a possible bacteriocinogenic effect.

2.5.9 Production of inhibitory substances by LAB in a meat system

In an earlier study by Burns (1987) it was shown that a strain of LAB isolated from ground beef produced an inhibitory substance. The identity of the producer strain, nature of the inhibitory substance and its activity on synthetic media and VP ground beef were tested. The LAB strain was active against a range of microorganisms but it did not produce inhibitory substance on synthetic media at pH below 6.0. Inhibition of *Streptococcus faecalis* by the LAB producer strain in ground beef was only seen when the producer strain

was inoculated into ground beef at high concentrations (10^9 CFU/g). The inhibitory effect seen was assumed to be mainly an acidity effect.

The previous study failed to demonstrate bacteriocin-like inhibitory substance production in ground beef. Therefore one of the objectives of the present study was to show production of the inhibitory substance by a selected LAB strain in refrigerated ground beef and also test the conditions necessary for the production of bacteriocin-like substance in broth.

3.MATERIAL AND METHODS

3.1 Preparation of ground beef

Freshly excised beef *semitendinosus* muscle was used to prepare samples of ground beef for use in the meat studies by aseptically trimming the muscle and grinding the inside portion in a sterile, manual meat grinder. Samples weighing 100 to 200 g were aseptically weighed into sterile plastic bags and stored at -70°C.

3.2 Bacterial cultures

The lactic acid bacteria used in this study were obtained from different sources:

a-Isolates obtained from vacuum packaged fresh beef (University of Alberta) included: UAL 3, 4, 26, 59, 72 and 86.

b-Isolates donated by Dr B. G. Shaw (AFRC Institute of Food Research, Langford, Bristol UK): UAL 7, 8, 9, 11, 12 and 16.

c-ATCC cultures: *Leuconostoc mesenteroides* ATCC 23368.

The "contaminating" microflora used in this study were either ATCC strains or bacteria that had been isolated in previous meat studies (University of Alberta) and included: *Staphylococcus aureus* (ATCC 25923), *Pseudomonas fluorescens* (ATCC 3525), *Escherichia coli* (meat isolate #1840) and *Klebsiella pneumoniae* (meat isolate #2).

3.3 Maintenance and culture of the strains

LAB were stored in cooked meat medium (CMM, Difco Laboratories, Detroit, MI) at 4°C and subcultured every 3 months. Before use in an experiment the cultures were grown in BM broth for 18-20 h at 25°C with one subculture in the same broth. BM broth is modified MRS broth as described by Wilkinson and Jones, (1977). Strains used as the "contaminating" microflora were kept on tryptic soy agar (TSA, Difco) slants at 4°C and subcultured monthly. Before use in an experiment they were subcultured twice in tryptic

soy broth (TSB, Difco) and incubated at the appropriate temperature for each strain (25 or 35°C).

Bacterial inocula for addition to the meat samples were prepared from a 20-h culture, centrifuged at 7,700 x g for 15 min and the cell pellet resuspended in 1 mL of 50 mM phosphate buffer (NaH_2PO_4-Na_2HPO_4, Fisher Scientific, Fair Lawn, NJ) at pH 7.0. This inoculum was used for the experiments unless otherwise stated. A 10 mL aliquot of culture of the LAB strain in BM broth was inoculated into 11 g of ground beef. The inoculum of the "contaminating" microflora consisted of an equal volume of each of the 4 strains and 40 µL was added to the ground beef sample.

3.4 Culture media

The broth and agar media used for this study are listed in Table 3.1. Media were prepared following the manufacturer's instructions or the directions given in the reference cited. The pH of Lactobacilli MRS agar (MRS, Difco) was adjusted to pH 5.6 with 85% lactic acid (Fisher Scientific). The pH of other media was adjusted with 1 N HCl (Specific gravity 1.19, BDH Chemicals, Toronto) or 1 N NaOH (Fisher Scientific). BM broth was used as the general culture medium for growth of LAB.

3.5 Inoculation of selected LAB in ground beef

3.5.1 Bacterial inoculum

The LAB used for this study were UAL3 and UAL59. UAL3 was chosen as a strain without a bacteriocin-like inhibitory activity but good growth capacity at low temperature. UAL59 produced a bacteriocin-like inhibitory substance, grew less efficiently at low temperature, but had a wide inhibitory spectrum against gram positive and gram negative bacteria. Other physiological and biochemical attributes of these LAB are shown in Table 3.2

3.5.2 Sample preparation

Ground beef prepared as described in section 3.1 was thawed and weighed aseptically (11 g) into Whirl-Pak bags (Nasco, Fort Atkinson, WI). Bacteria were inoculated into the meat according to the following scheme:

Sample	Inoculum size (final concentration)
Control	uninoculated
"Contaminating" microflora	10^3 CFU/g
UAL3	10^3 CFU/g
UAL59	10^3 CFU/g
UAL3 with "contaminating" microflora	10^3 CFU/g
UAL3 with "contaminating" microflora	10^6 CFU/g
UAL59 with "contaminating" microflora	10^3 CFU/g
UAL59 with "contaminating" microflora	10^6 CFU/g

The inoculum was mixed thoroughly by manipulating the meat and bacterial inoculum in the Whirl-Pak bag. The meat was flattened to an area of 4 x 10 cm in the Whirl-Pak bag, evacuated and sealed. Sets of 8 bags representing each treatment were placed in tri-layer "Vacpac" bags (Cryovac, O_2 transmission 30-50 $cm^3/m^2/h$, 22°C, 1 atm), vacuum packaged and sealed. Storage and sampling was done as indicated:

Temperature	Sampling days
10°C	every 2 days up to 28 days
4°C	every 4 days up to 42 days
1°C	every 7 days up to 42 days

Table 3.1. Selective and nonselective media used and their supplier or reference.

Name	Abbreviation,Supplier/Reference
Non-selective Broth Media	
Cooked Meat Medium	CMM, Difco[1]
Basal Medium (modified MRS broth)	BM, Wilkinson and Jones (1977)[2]
All Purpose Tween Agar	APT, Difco[1]
Tryptic Soy Broth	TSB, Difco[1]
Non-selective Plating Media	
Tryptic Soy Agar	TSA, Difco[1]
Selective Plating Media	
Streptomycin, Thallous Acetate, Actidione Agar	STAA, Gardner (1966)[2,3]
Baird-Parker Agar	Difco[1]
Cephaloridine, Fusidic Acid, Cetrimide Agar	CFC, Mead and Adams (1977)[2,3]
Violet Red Bile Agar	VRBA, Difco[1]
MacConkey Inositol Carbenicillin Agar	MCIC, Bagley and Seidler (1978)[2,3]
Lactobacilli MRS Agar (pH 5.6)	MRS, Difco[1]

[1] Difco Laboratories, Detroit, MI.

[2] Prepared from Difco ingredients and chemicals from Fisher Scientific, Fair Lawn, NJ.

[3] Antimicrobial agents: cephaloridine, fusidic acid sodium salt, cetrimide (hexadecyl trimethyl ammonium bromide) from J.T. Baker Chemical Co., Phillipsburg, NJ; actidione (cycloheximide) and streptomycin sulfate from Sigma Chemical Co., St. Louis, MO; thallous acetate from Fisher Scientific.

Table 3.2. Physiological and biochemical attributes of LAB strains UAL3 and UAL59.

		UAL3	UAL59
Growth in			
NaCl	5%	+	+
	7.5%	+	+
	10%	+	±
200 ppm $NaNO_2$		+	+
200 ppm $NaNO_2$ + 5% NaCl		+	+
Growth at low temperature			
4°C		2 days	±
1°C		3 days	±
pH			
Growth at pH 3.9		-	+
Final pH in La [1]broth (7 days)		3.9	3.7
Inhibitory substance production at low temperature			
4°C		-	-
1°C		-	-
Inhibitory substance production at low pH (deferred antagonism)[2]			
pH 5.0		+	+
pH 4.5		+	+

1 La = MRS broth without phosphate, sodium citrate replacing ammonium citrate, adjusted to pH 6.7 - 6.8
2 acidity effect or bacteriocin - like substance inhibition

3.5.3 Sample testing

Organoleptic characteristics of color and odor of ground beef samples was evaluated as an indication of possible consumer acceptance of the meat during storage. For odor a scoring system was used, in which the following scores were used:

1+ slightly sour

2+ sour

3+ strongly sour

4+ very sour, with off odors

The color of the meat was also described.

For the microbiological analysis, samples were tested at time zero, immediately after inoculation and at the indicated storage intervals (section 3.5.2). Sterile 0.1% peptone water (99 mL) was added to the meat in the Whirl-Pak bag and blended in a Colworth Stomacher (Model 400, A. J. Seward, 3 Cavendich Rd., Bury St. Edmund, Suffolk, U.K.) for 2 min. Dilutions were made in 0.1% peptone water and surface plated onto the appropriate selective and nonselective media to determine the viable bacterial counts.

For the inoculated LAB strains, MRS agar adjusted to pH 5.6 was used. TSA was used as a general growth medium to determine the aerobic plate count of the uninoculated control sample. The selective media included: Streptomycin, Thallous Acetate, Actidione Agar (STAA, Gardner, 1966) for presumptive detection of *B. thermosphacta;* Baird-Parker Agar (Difco) for *S. aureus;* Cephaloridine, Fusidic acid, Cetrimide Agar (CFC, Mead and Adams, 1977) for *P. fluorescens*; Violet Red Bile Agar (Difco) for *E. coli*; and MacConkey, Inositol, Carbenicillin Agar (MCIC, Bagley and Seidler, 1978) for *K. pneumoniae*. Incubation of MRS agar was done anaerobically (anaerobic jar flushed twice with a mixture of 10% CO_2 and 90% N_2) and incubated at 25°C for 72 h. CFC and STAA agar plates were incubated aerobically at 25°C for 48 h, while VRBA was incubated at 44.5°C for 24 h. MCIC and Baird-Parker plates were incubated at 35°C for 24 and 48 h,

respectively. Colonies of *P. fluorescens* growing on CFC agar were checked for fluorescense under UV light (Fotodyne Incorporated, USA).

The pH of the samples was measured in the 10^{-1} dilution of the meat sample using an Orion combination electrode (Orion Research Inc., Cambridge, MA) connected to a Fisher Accumet^R pH meter Model 600.

3.6 Preparation of pH adjusted ground beef

Ground beef prepared as described in section 3.1 was mixed with 1M phosphate buffer (Na_2HPO_4-NaH_2PO_4) at pH 6.5 to give a final concentration of 0.1M in the meat or with $CaCO_3$ (Fisher Scientific) to give a final concentration of 10% (w/w) in the meat. The pH of the adjusted meat samples was 6.5 for both systems. LAB strains UAL 3, 8 and 59 and the "contaminating" microflora were inoculated into the ground beef to give a final concentration of each organism of 10^5 CFU/g. Samples were stored in VP at 15°C and sampled after 2 and 5 days of storage. Viable cell counts and pH of the meat were checked at time zero, and on each sampling day. Plating was done on the appropriate selective and nonselective media described in section 3.5.2.

3.7 Addition of catalase to inoculated ground beef

Ground beef prepared as described in section 3.1 was mixed with $CaCO_3$ (10% w/w), weighed in 11 g portions into Whirl-Pak bags and γ–irradiated for 22 hours (7 kGy) in a Gamma cell 220 (Atomic energy of Canada Ltd., Ottawa, Ont.). LAB strain UAL3 inoculum was prepared as described in section 3.3 and added to give a final concentration of 10^7 CFU/g in the meat. The contaminating microflora was inoculated to give a final concentration of each organism of 10^5 CFU/g. Filter sterilized catalase (from Bovine liver, Sigma Chemical Co., St Louis, MO) was added to give a concentration of 100 units/g of meat. The ground beef samples were VP, stored at 15°C, and tested after 24 and 48 h. As a control for catalase activity in the incubated ground beef, the enzyme was added to

uninoculated meat samples and catalase activity checked after incubation by adding a 3% solution of hydrogen peroxide (Fisher Scientific).

3.8 Ground beef adjusted with lactic or hydrochloric acids

Meat samples were prepared as described in section 3.1 and the pH was adjusted to 4.5 and 5.0 with 8.5% lactic acid (Fisher Scientific) and 1N HCl. Samples were γ-irradiated for 22 h (7 kGy) and inoculated with the contaminating microflora to give a final concentration of 10^5 CFU/g of meat. Vacuum packaged samples were stored at 4°C and sampled every second day for 10 days. At each sampling time viable bacterial counts of the contaminating microflora were determined on selective media described in section 3.5.2.

3.9 Methods for detection of inhibitory substances produced by LAB

Conditions necessary for production of inhibitory substance by LAB were tested for 11 different producer strains isolated from meats.

3.9.1 Direct antagonism

Test and indicator microorganisms were grown simultaneously on the agar plate (Tagg *et al.*, 1976). Spot inocula of the producer organisms that had been grown in BM broth at 25°C for 20 h were inoculated onto APT, MRS and TSA agar plates using a replicating inoculator (Cathra International Systems for the Microbiologist, Diagnostic Equipment, Inc., St. Paul, MN) and dried in a laminar flow cabinet (The Baker Co., Inc., Sanford, ME). Plates inoculated with potential producer strains were overlayered with 6 mL of the respective soft agar (0.75% agar) that had been inoculated with the indicator strain at 10^6 CFU/mL using 0.6 mL of a 10^{-1} dilution of the indicator strain grown in BM broth at 25°C for 20 h. Inoculated and overlayered plates were incubated at 25°C for 24 h in an atmosphere containing 10% CO_2 and 90% N_2. The indicator lawn was examined for zones of inhibition surrounding each producer strain.

3.9.2 Deferred antagonism

Producer strains were spotted onto agar plates using the inoculator and incubated under anaerobic conditions for 24 h at 25°C. Subsequently the plates were overlayered with the indicator strains, as described for direct antagonism, and incubated under anaerobic conditions at 25°C for an additional 24 h. The indicator lawn was examined for zones of inhibition surrounding each producer strain.

3.9.3 Bacteriocin detection in broth supernatant

A 24-h culture of the producer strain grown in BM broth at 25°C was centrifuged at 7,700 x g for 15 min. The supernatant was adjusted to pH 6.5 with 1N NaOH and remaining LAB cells were inactivated by heating the supernatant at 62°C for 30 min. Wells were cut into APT plates with a 7 mm sterile cork borer. The bottom of each well was sealed with 2 drops of APT agar. A 60 µL aliquot of pH adjusted (6.5) supernatant was placed in each well, allowed to diffuse into the agar at room temperature for 4 h and then dried in the laminar flow cabinet for 15 min. An 8 mL overlay of inoculated APT soft agar was added to the plate, as described for direct antagonism. The inoculated plates were incubated under anaerobic conditions at 25°C for 24 h. The indicator lawn was checked for zones of inhibition surrounding each well.

3.9.4 Test for inhibition due to bacteriophage

The reverse side inoculation technique was used, in which the test supernatant was inoculated into the agar by the well technique, as described in section 3.9.3, but after drying the agar was inverted into the cover of the petri dish. The reverse side of the agar was covered with the indicator lawn as described in section 3.9.1. Plates were incubated anaerobically at 25°C for 24 h and checked for zones of inhibition around the wells.

3.9.5 Bacteriocin production on solid media using the method of Barefoot and Klaenhammer, (1983)

Five APT plates were surface inoculated with 0.2 mL of a 20-h culture of the producer strain and incubated anaerobically at 25°C for 48 h. The agar was removed from the plates, placed into a sterile Whirl-Pak bag and weighed. An equal weight of sterile 3 mM phosphate buffer (NaH_2PO_4-Na_2HPO_4 at pH 7.0) was added, the agar-buffer mixture was crushed in a Stomacher blender for 45 sec and left to equilibrate at 1°C for 24 h. The agar-buffer mixture was prefiltered through Whatman no. 1 filter paper under vacuum, followed by centrifugation at 7,700 x g for 15 min. The supernatant was adjusted to pH 6.5 with 1N NaOH and filter sterilized through a 0.45 µm Nucleopore filter (Nucleopore Corporation, Pleasanton, CA). The activity of the extracts was checked by the agar well method described in section 3.9.3. As a variation of this method the producer strains were grown in APT with 0.5% agar. A 20 mL aliquot of the soft APT agar was mixed with 0.2 mL of a 20-h culture of the strain grown in BM broth. The filtration step through Whatman filter paper was omitted and replaced by two clarifying centrifugations.

3.10 Concentration of the inhibitory substance

The inhibitory substance from the broth supernatant (section 3.9.3) or the culture extract (section 3.9.5) was concentrated using an Amicon ultrafiltration apparatus (Amicon Division, W.R. Grace & Co., 24 Cherry Hill Drive, Danvers, MA) using a membrane (Diaflo ultrafilters PM 10, Amicon) with a 10, 000 MW cut off. After concentration of the supernatant (10 to 15 times) the solution was adjusted to pH 6.5 and vegetative cells were inactivated by heating (62°C for 30 min).

3.11 Determination of arbitrary activity units of inhibitory substance

Arbitrary activity units (AU) of the inhibitory substance per mL were determined by the spot on lawn technique (Barefoot and Klaenhammer, 1983). APT agar was

overlayered with 6 mL of soft APT agar containing the sensitive indicator strain at 10^6 CFU/mL. A series of twofold dilutions of the prepared inhibitory solution was made in 50 mM phosphate buffer at pH 7.0 and 20 µL of each dilution was spotted onto the prepared lawn. The AU/mL was determined from the reciprocal of the highest dilution at which complete inhibition was observed (i. e. clear zone of inhibition with sharp edges).

3.12 Characterization of the nature of the inhibitory substance

To test for the proteinaceous nature of the inhibitory substance an aliquot of the pH adjusted and sterilized supernatant was treated with filter sterilized protease from *Bacillus subtilis* (1 mg/mL, Sigma). Inactivation was checked using a 20 µL aliquot of the protease treated and control samples by the spot on lawn technique described in section 3.11.

3.13 Production of inhibitory substance by UAL8 in heat treated ground beef

3.13.1 Extraction of inhibitory substance from heated and raw meat

Ground beef was prepared as described in section 3.1, 1.25 g of the meat with 1% $CaCO_3$ (w/w) was weighed into a sterile test tube and 10 mL of sterile, distilled water added. Half of the tubes containing the meat was autoclaved (121°C for 20 min). The pH of the meat before inoculation was 6.5. Also, 20 g samples of ground beef containing 1% $CaCO_3$ (w/w) were placed in Whirl-Pak bags. LAB strain UAL8 was grown in BM broth at 25°C for 18 to 20 h, then centrifuged (7,700 x g) for 15 min. The pellet was resuspended in 1 mL of sterile 50 mM phosphate buffer at pH 7.0. From this suspension, 100 mL were added to each 10 mL of meat suspension or 200 µL to each 20 g sample of meat in a Whirl-Pak bag. The inoculated ground beef samples were placed in vacuum bags (O_2 transmission 40.7 $cm^3/m^2/24$ h at 22°C, 1 atm, Unipac, Edmonton, Alberta; this type vacuum bag was used in the successive meat inoculation experiments), VP and stored at 15°C for 48 h. CMM and BM broth were inoculated with strain UAL8 as positive controls

for production of the inhibitory substance. BM broth or CMM supernatant was centrifuged (7,700 x g) for 15 min and heat treated (62°C for 30 min). Extraction of the inhibitory substance from ground beef was done by adding an equal volume (20 mL) of 50 mM phosphate buffer at pH 7.0. The samples were centrifuged and heat treated (62°C for 30 min). Activity was tested by the spot on lawn technique (section 3.11) against indicator UAL9.

3.13.2 Production and stability of the inhibitory substance in different concentrations of ground beef

A 0.5 mL aliquot of 50 mM phosphate buffer at pH 7.0 was added to 1 g of ground beef and twofold dilutions up to 1/16 were made with added buffer. To each dilution, 0.1 mL of inhibitory supernatant from strain UAL8 (20-h culture in BM broth grown at 25°C, adjusted to pH 6.5 and heat treated) was added. The dilutions were equilibrated at 15°C for 3 h and clarified by centrifugation (7,700 x g for 15 min). The pH of the supernatant was checked, adjusted to pH 6.5 when necessary and heat treated (62°C for 30 min). Strain UAL8 was also added to dilutions of ground beef to check for production of the inhibitory substance. Samples of ground beef (2 g) were mixed with 1% $CaCO_3$ (w/w), placed into test tubes and 1, 2, 4, 8 and 16 mL of sterile, distilled water was added. Also, 10 g of ground beef were placed in Whirl-Pak bags. A 24-h culture of strain UAL8 grown in BM broth at 25°C was centrifuged, the pellet resuspended in 1 mL of 50 mM phosphate buffer and different amounts of the culture added to each test tube, to achieve 10 mL inoculum per gram of final suspension according to the following protocol:

GROUND BEEF	DISTILLED WATER	UAL8 (10^9 CFU/mL)
10 g	——	100 mL
2 g	1 mL	negative control
2 g	1 mL	30 mL
2 g	2 mL	40 mL
2 g	4 mL	60 mL
2 g	8 mL	100 mL
2 g	16 mL	180 mL

The ground beef samples in Whirl-Pak bags were VP and all samples were stored at 15°C for 48 h. Liquid samples were centrifuged, the supernatant adjusted to pH 6.5 and heated at 62°C for 30 min or filter sterilized (0.45 µm, Millipore Corporation, Bedford, MA). The activity in the supernatants was checked by the spot on lawn technique (section 3.11). The ground beef without added liquid was left to equilibrate in 50 mM phosphate buffer for 1 h at 1°C and treated in the same manner as that described for the liquid samples above.

3.14 γ-irradiation of inoculated ground beef

3.14.1 Extraction with buffer during irradiation

Ground beef inoculated with UAL8 and stored for production of inhibitory substance was irradiated to inactivate the producer strain. The meat samples stored in a Whirl-Pak bag were mixed with an equal volume of 50 mM phosphate buffer at pH 7.0, blended in the Stomacher for 2 min and γ-irradiated.

3.14.2 Freezing of the meat sample

To improve the extraction procedure, after adding the phosphate buffer to the ground beef sample in the Whirl-Pak bag, samples were transferred into a test tube and

frozen at -70°C for 30 min before irradiation. During irradiation the samples were kept in a beaker with ice.

3.15 Production of inhibitory substance by strain UAL8 in ground beef at 4°C

Ground beef was prepared as described in section 3.1. Sterile $CaCO_3$ was added to the meat during grinding to a final concentration of 1% (w/w). Ten gram samples of ground beef were weighed into sterile Whirl-Pak bags and treated as follows:

(a) Ground beef with 1% $CaCO_3$ inoculated with UAL8

(b) Ground beef without $CaCO_3$ inoculated with UAL8

(c) Ground beef with 1% $CaCO_3$ (control)

(d) Ground beef without $CaCO_3$ (control)

(e) CMM broth inoculated with UAL8 (positive control)

Test samples were stored at 4°C for 15 days and tested at 5-day intervals. Growth of strain UAL8 in the meat samples was monitored on MRS agar before γ-irradiation. Extraction in buffer was done as described in section 3.14.1 and 3.14.2. After irradiation, the samples were centrifuged. The activity of the supernatants was tested by the spot on lawn technique (section 3.11).

3.16 Stability of the inhibitory substance in ground beef stored at 4°C

A 4 g sample of aseptically prepared ground beef (section 3.1) was placed in a Whirl-Pak bag and 1 mL of 10 times concentrated UAL8 supernatant (1600 AU/mL) was added to give a final concentration of 400 AU/mL. The ground beef and the UAL8 supernatant were mixed by manipulation of the meat and then VP. The samples were stored at 4°C for 5 days and tested at 12 h intervals. Extraction of the inhibitory substance from the meat was done by adding 4 mL (w/v) of sterile 50 mM phosphate buffer at pH 7.0, allowing the mixture to equilibrate at 1°C for 22 h, centrifuging and filter sterilizing

the supernatant through an Acrodisc membrane (0.45 µm Gelman Science Inc., Ann Arbor, MI). A series of twofold dilutions of the extract was made to determine the activity present in the supernatants (AU/mL) by the spot on lawn technique (section 3.11).

In this experiment, a positive result was recorded when slight inhibition was seen, i.e. the last dilution before complete overgrowth of the indicator lawn. This dilution was used for calculation of the AU/mL. This interpretation of a positive result differs from the one used in broth (section 3.17) because the activity recovered from ground beef was always less than from a broth culture.

3.16.1 Monitoring production of the inhibitory substance at 4°C

Ground beef was prepared as described in section 3.1 with 1% $CaCO_3$ (w/w) added during grinding. An 11 g sample was weighed into Whirl-Pak bags and inoculated with a UAL8 inoculum prepared as described in section 3.1. The samples were VP and stored at 4°C.

Plate count and activity of the inhibitory substance were determined at 2-day intervals for 12 days. Viable cell counts were determined by plating on MRS agar. Extraction of the inhibitory substance was done with 50 mM phosphate buffer at pH 7.0. The meat and buffer were equilibrated at 1°C for 20 h. Samples were centrifuged and the pH was checked and adjusted to 6.5 where necessary. The samples were filter sterilized through an Acrodisc membrane (0.45 µm). The activity of the extract was tested by the spot on lawn technique (section 3.11). Inhibitory activity was interpreted as described in section 3.16.

3.17 Production of inhibitory substance by strain UAL8 in broth under different conditions of pH, carbohydrate concentration and storage temperature

The experiment was done using BM broth modified for the required test conditions. Variables for this experiment were:

pH	6.0, 5.5 and 5.0
Carbohydrate concentration (glucose)	2% and 0.2%
Storage temperature	25, 10, 4 and 1°C

The pH of the broth was adjusted with 85% lactic acid.

The modified broths were inoculated with a 24-h culture of strain UAL8 resuspended in 10 mL of 50 mM phosphate buffer at pH 7.0 to give a concentration of 10^8 CFU/mL. A 1% inoculum was added to each tube to give a final concentration of approximately 10^6 CFU/mL. The broth was held at the storage temperatures and sampled as follows:

25°C every day for 5 days

10°C every 2 days for 10 days

4°C every 4 days for 20 days

1°C every 4 days for 20 days

At each sampling time the pH of the culture was measured, viable counts were determined on MRS agar and inhibitory activity was checked. To determine the inhibitory activity, culture supernatants were adjusted to pH 6.5 heated at 62°C for 30 min and tested by the spot on lawn technique (section 3.11). The AU/mL were calculated from the highest dilution showing complete clearing of the indicator lawn.

4.RESULTS

4.1 Influence of LAB strain UAL3 and UAL59 on the growth and survival of a "contaminating" microflora inoculated into vacuum packaged ground beef stored at different temperatures

4.1.1 Temperature effect on growth of selected LAB inoculated into ground beef

Inoculated ground beef stored at 10, 4 and 1°C showed differences in the ability of the LAB strains to grow. At 10°C strain UAL59 grew to 10^7 CFU/g in 30 days. At 4 and 1°C poor growth of UAL59 was even more marked. As shown in Figure 4.1 at 1°C there was less than 10-fold increase in count of UAL59 inoculated at 10^3 CFU/g during 42 days of storage. In meat inoculated with this LAB at 10^3 CFU/g, a large number of small, pinpoint colonies grew on MRS agar together with the typical inoculated LAB colonies. Strain UAL3 inoculated at 10^3 CFU/g into ground beef and incubated at 10 and 4°C reached 10^8 CFU/g after 11 and 14 days, respectively. At 1°C a maximum population of 10^7 CFU/g was achieved after 14 days, with no further increase during storage up to 42 days.

Growth of adventitious lactic acid bacteria is shown in Figure 4.2. LAB counts increased to 10^7 CFU/g during 7 days storage at 10°C, while at 4 and 1°C it took 14 days to reach this count. Minimum pH was reached after 21 days incubation at 4°C (Figure 4.3). The growth rates of the adventitious LAB and UAL59 at 10°C were similar. On the other hand, UAL59 inoculated at 10^3 CFU/g only grew to 10^5 CFU/g at 4°C and to 10^4 CFU/g at 1°C, while the adventitious LAB reached counts of 10^7 CFU/g in 14 days at both temperatures. UAL3 showed a comparable growth rate with that of the adventitious LAB at all three storage temperatures.

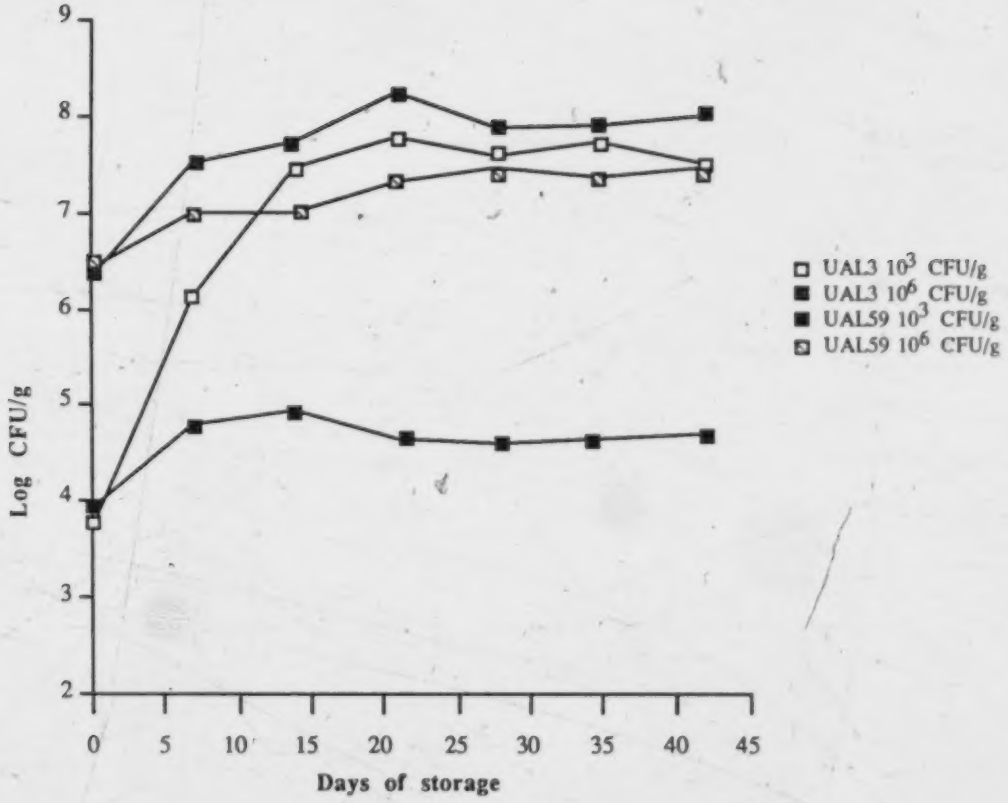


Figure 4.1. Effect of inoculum level on growth of LAB strains UAL3 and UAL59 in vacuum packaged ground beef during storage at 1°C.

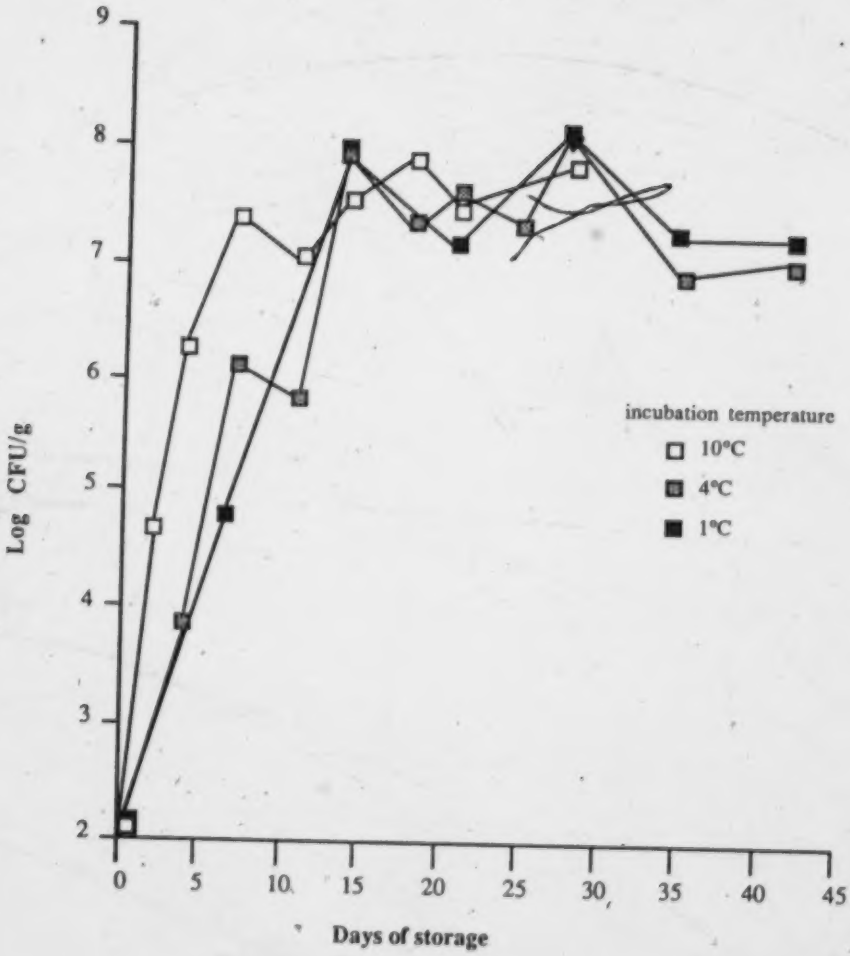


Figure 4.2. Growth of adventitious lactic acid bacteria in vacuum packaged ground beef stored at 10, 4, and 1°C.

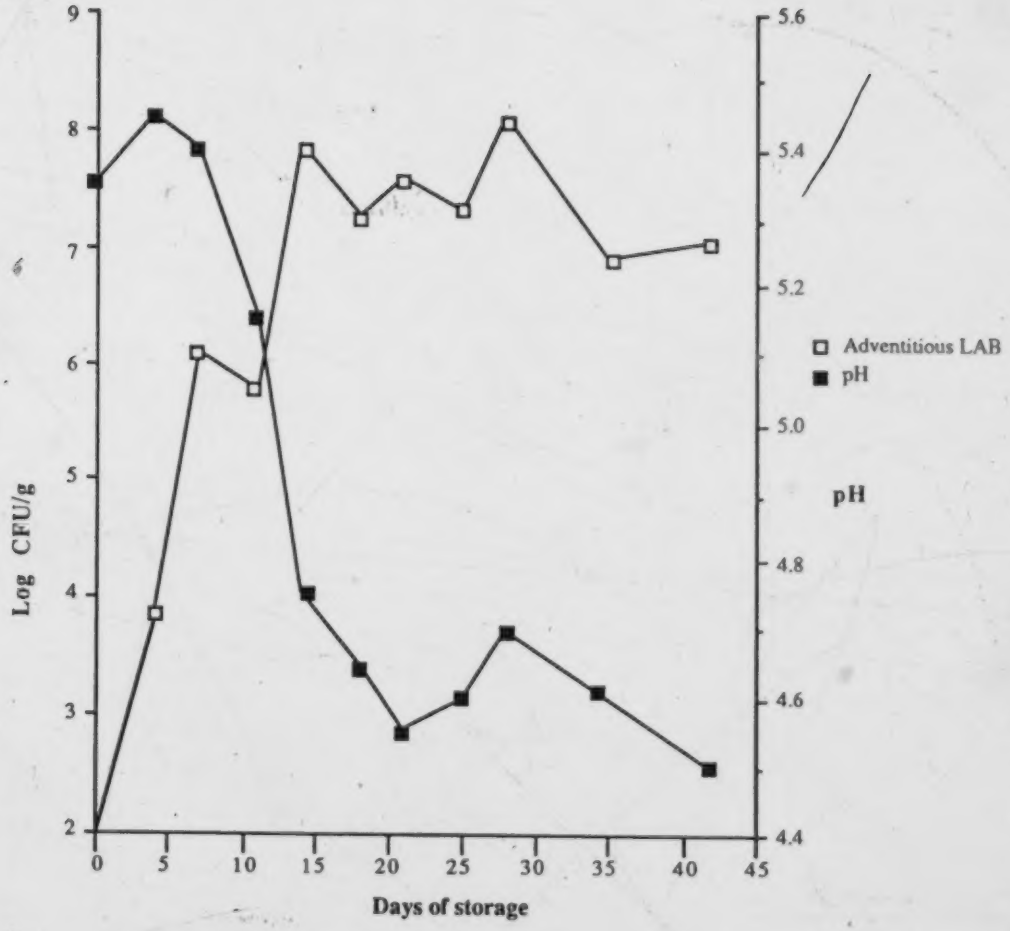


Figure 4.3. Growth of adventitious lactic acid bacteria and change in pH of vacuum packaged ground beef stored at 4°C.

4.1.2 Growth of *B. thermosphacta* in VP ground beef

Adventitious *B. thermosphacta* enumerated on STAA agar reached counts of 10^4 CFU/g in the control samples at 10°C during the first 7 days of storage. A sharp decline to undetectable levels (<100 CFU/g) occurred after 11 days of storage. At 4°C, maximum counts of 10^3 CFU/g were recorded after 11 days, followed by a rapid decline in numbers by 14 days. *B. thermosphacta* could be recovered from samples stored at 1°C up to 42 days. Data in Table 4.1 show that in meat with strain UAL3 added at low inoculum, *B. thermosphacta* was detected up to 28 days during storage at 1°C, while at the higher inoculum level *B. thermosphacta* was not detected in meat samples at any time during storage.

4.1.3 Survival of an added "contaminating" microflora in the ground beef

The "contaminating" microflora consisted of *S. aureus*, *P. fluorescens*, *E. coli* and *K. pneumoniae*. It was determined that these bacteria grew associatively without the production of antagonistic substances that were inhibitory to their survival or growth. Each of the contaminating bacteria was inoculated into ground beef samples at 10^3 CFU/g. The survival of the inoculated microflora in ground beef was studied at three temperatures and in the presence of added LAB.

From the data shown in Table 4.2 it can be seen that *S. aureus* was the least affected by the added LAB. There was a slow decrease in viability, particularly at 1°C, but even after 42 days of storage survivors could still be detected. The survival of *P. fluorescens* was markedly affected by high concentrations of LAB. However, when LAB strains UAL 3 and 59 were added at 10^3 CFU/g, *P. fluorescens* first increased in numbers, and then declined slowly during the remainder of the storage period. The control sample stored at 4°C with added contaminating microflora, but without added LAB, showed similar results to those obtained with UAL59 inoculated at the lower concentration.

Table 4.1. Effect of incubation temperature and lactic acid bacteria inoculum on survival of *Brochothrix thermosphacta* in vacuum packaged ground beef.

Added Lactic Acid Bacteria	Storage temperature		
	10°C	4°C	1°C
	Days to nondetectable level (<100 CFU/g)		
Control (no added microflora)	9	14	42
UAL3 (10^3 CFU/g)	4	11	28
UAL3 (10^6 CFU/g)	ND [1]	ND	ND
UAL59 (10^3 CFU/g)	4	ND	ND
UAL59 (10^6 CFU/g)	4	ND	21

[1] not detected on STAA agar at 10^2 CFU/g

Table 4.2. Time to achieve maximum population of lactic acid bacteria, pH <4.7 and elimination (<10^2 CFU/g) of contaminating microflora in vacuum packaged ground beef.

	UAL3		UAL59		Control [a]
Inoculum	**10^3/g**	**10^6/g**	**10^3/g**	**10^6/g**	
Storage temperature 10°C					
LAB (max. 10^7/g)	4	2	18	2	7
pH <4.7	9	9	11	9	9[b]
S. aureus	21	21	21	28	>9
P. fluorescens	28	7	28	4	9
E. coli	21	11	28	11	>9
K. pneumoniae	28	21	21	11	>9
Storage temperature 4°C					
LAB (max 10^7/g)	7	4	14 [c]	7	14
pH <4.7	14	14	14	14	14
S. aureus	42	35	42	35	>21
P. fluorescens	25	4	25	18	>21
E. coli	28	11	21	11	18
K. pneumoniae	28	35	25	14	>21
Storage temperature 1°C					
LAB (max 10^7/g)	14	7	7 [d]	7	14
pH <4.7	35	35	35	35	28
S. aureus	>42	>42	>42	>42	>42
P. fluorescens	42	7	42	21	42
E. coli	21	14	35	14	35
K. pneumoniae	21	28	21	28	42

a adventitious LAB or added "contaminating" microflora without added LAB
b only tested to maximum of 9 days storage
c maximum 10^5/g
d maximum 10^4/g

However, data in Figure 4.4 show a marked difference in survival of *P. fluorescens* between the control and the higher LAB concentration for both strains of LAB. In the presence of strain UAL3, *P. fluorescens* was isolated at time zero, whereas with strain UAL59 it was isolated up to 11 days of storage at 4°C.

The survival of *E. coli* at the three incubation temperatures is also shown in Table 4.2 were the death rate with an inoculum of 10^3 CFU/g was slower than with an inoculum of 10^6 CFU/g for both LAB strains. This was especially noticeable at 1°C, where even after 30 days storage, *E. coli* could still be detected at 10^3 CFU/g of UAL59. At the higher LAB concentration there was a sharp decline in the number of *E. coli*, so that after 14 days at the three storage temperatures and with both LAB strains they could no longer be detected. *E. coli* in the control samples at 4°C survived for 18 days (see Figure 4.5).

K. pneumoniae showed a similar pattern of survival to *E. coli* at the three storage temperatures, except for UAL3 at 10^6 CFU/g were a longer survival of *K. pneumoniae* was seen. When comparing the survival of *K. pneumoniae* at 4°C with that in the control sample without added LAB (see Figure 4.6) it can be seen that at the higher concentration of UAL59, *K. pneumoniae* could not be isolated, while in the uninoculated control sample it survived up to 21 days. Different patterns of inhibition were observed for the contaminating strains, depending of the strain of LAB added and its concentration. The contaminating microflora survived longer in the controls than in the test samples with the added LAB. This was especially noticeable for *E. coli* and *P. fluorescens* in the presence of UAL59 at 10^6 CFU/g.

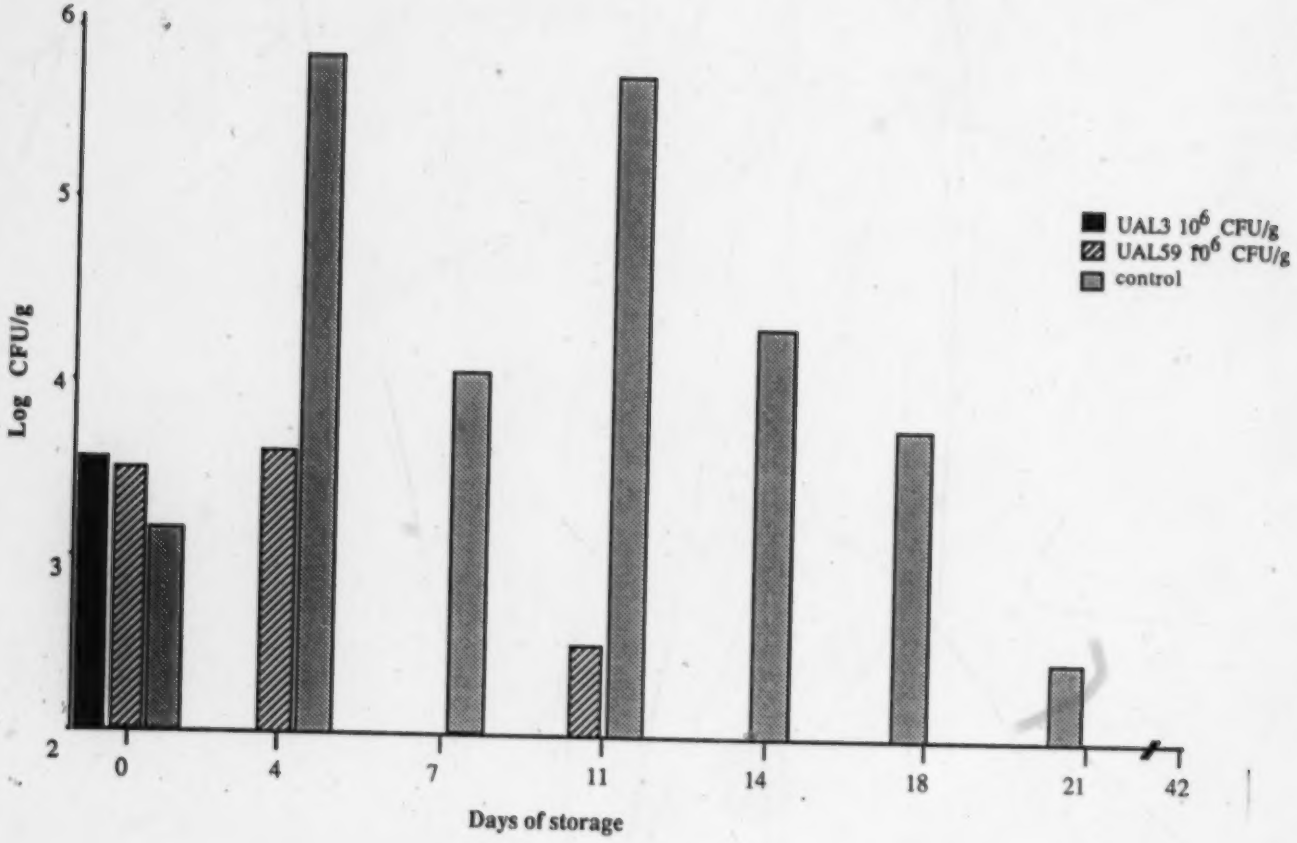


Figure 4.4 Survival of *Pseudomonas fluorescens* in vacuum packaged ground beef at 4°C in the presence of LAB strains UAL3 and UAL59 inoculated at 10^6 CFU/g.

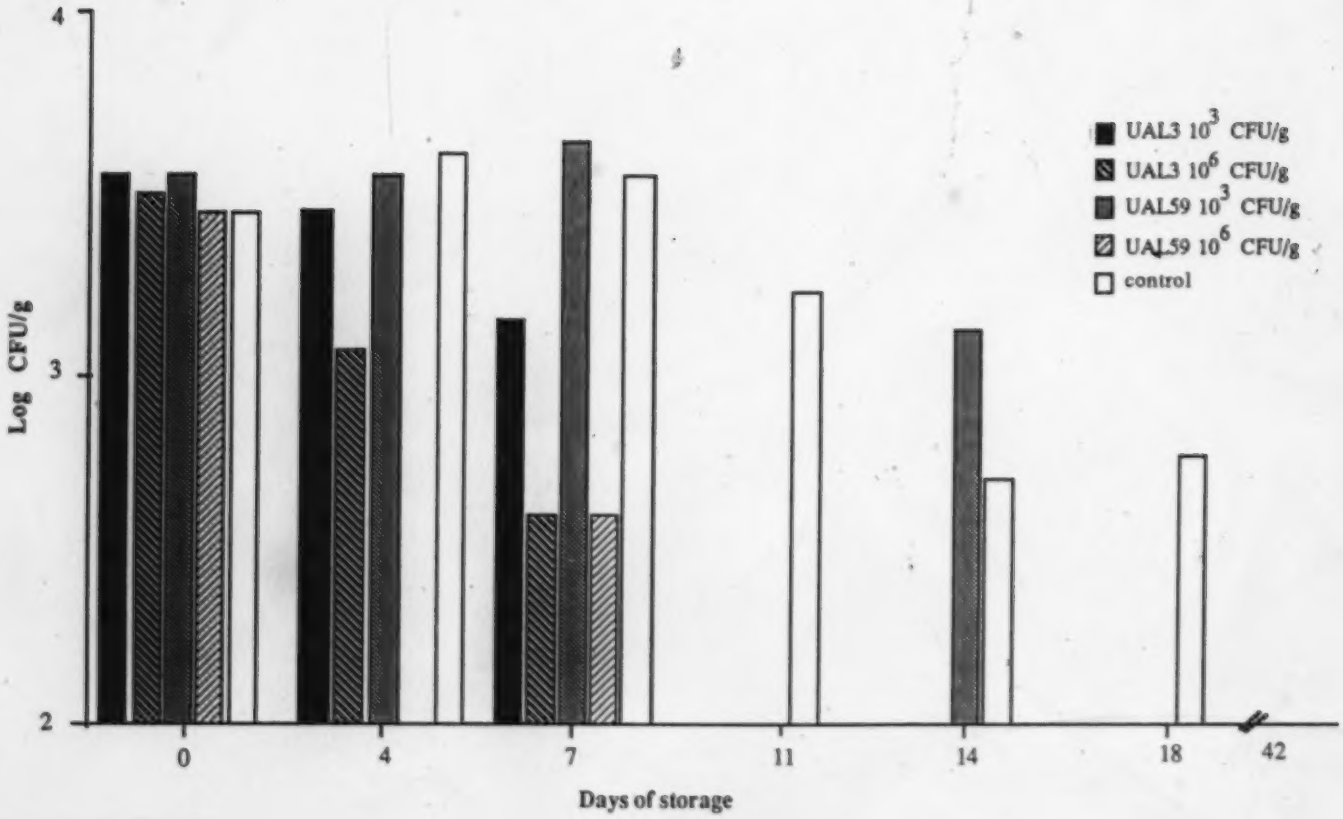


Figure 4.5. Survivial of *Escherichia coli* in vacuum packaged ground beef at 4°C in the presence of LAB strains UAL3 and UAL59 inoculated at two different concentrations.

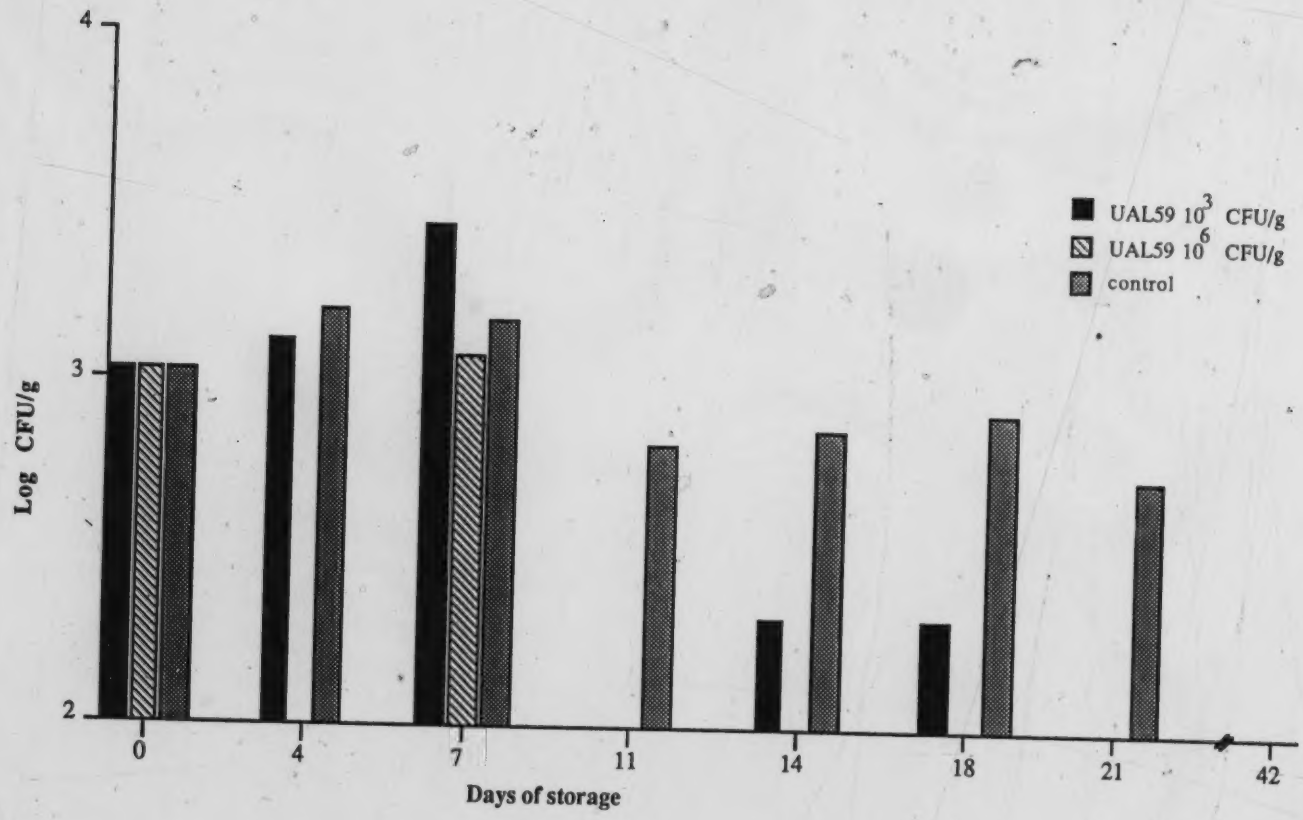


Figure 4.6. Survival of *Klebsiella pneumoniae* in vacuum packaged ground beef at 4°C in the presence of LAB strain UAL59 inoculated at two different concentrations.

4.1.4 Changes in meat pH during storage

The pH of the meat in the control samples dropped from 5.3 to 4.6 within 9 days for samples stored at 10°C. At 4°C this pH change occurred within 18 days, with a sharp decline occurring between 7 and 14 days coinciding with logarithmic growth of the adventitious LAB (see Figure 4.3). The pH was 4.6 after 20 days of storage at 4°C. The rate of drop in pH was similar for samples inoculated with LAB at either inoculum level (see Table 4.2). It was also observed that after the added LAB reached maximum population (10^7 to 10^8 CFU/g) the pH continued to decline independent of the strain of LAB inoculated. The survival of the contaminating microflora related to changes in pH of ground beef is shown in Figures 4.7 and 4.8.

4.1.5 Organoleptic characteristics of vacuum packaged ground beef during storage

Color of the VP meats did not compare favorably with oxygenated, retail ground beef. Samples were described as varying from light brown with pink spots to grey. Most samples between 4 and 9 days of storage had a grey color except for UAL59 at 10^6 CFU/g which had a pink color for approximately 18 days. The odor scores for the samples shown in Table 4.3 indicate that there were no differences in degree of sourness of the meat between inoculum levels of LAB. At 10 and 4°C, UAL3 produced sour odor sooner than UAL59, but this difference was not seen at 1°C. The meat samples tended to have sour smell after approximately 10 days of storage at the three temperatures.

4.1 6 Effect of acidity on contaminating microflora in ground beef

The addition of a chemical to buffer ground beef ($CaCO_3$ and phosphate buffer) enabled meat pH to be maintained above pH 6.0 (only with $CaCO_3$). Under these conditions and with added LAB it was seen that the contaminating microflora was not inhibited, indicating that part of the loss of viability detected in the previous experiment was

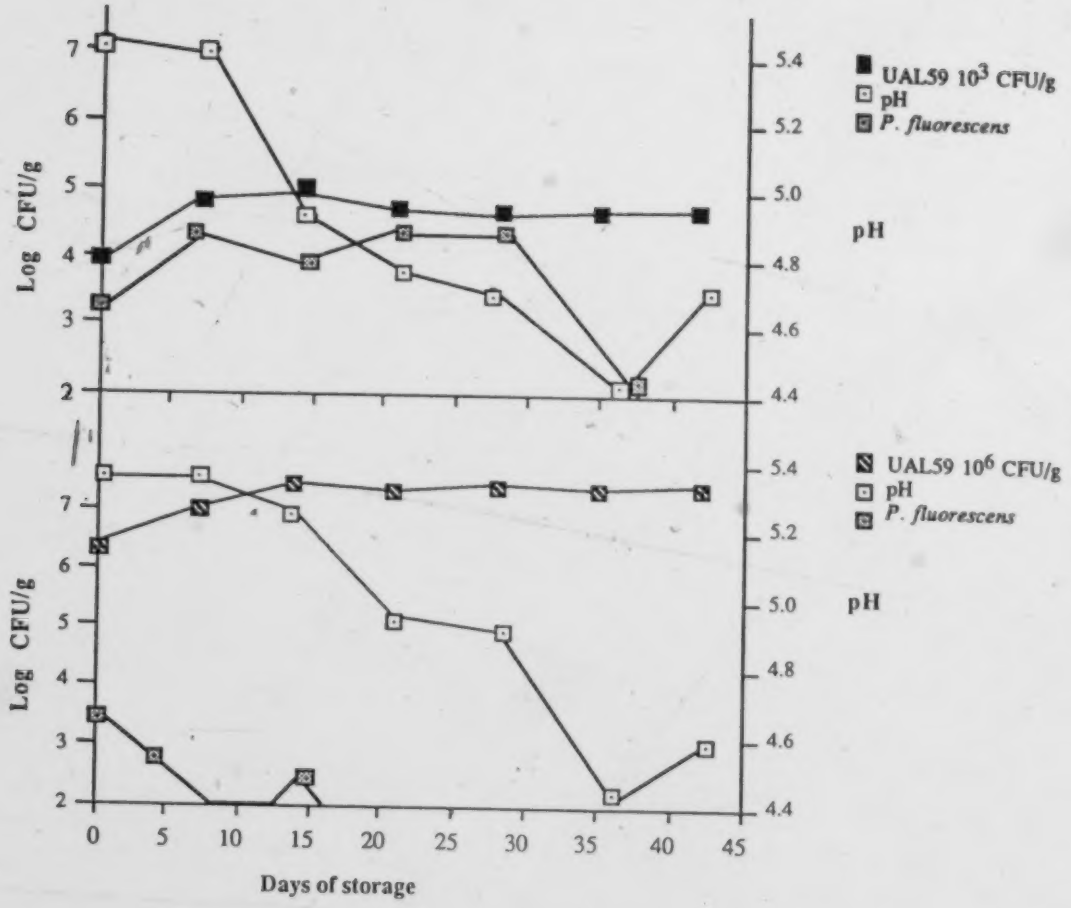


Figure 4.7. Survival of *Pseudomonas fluorescens* at 1°C in the presence of LAB strain UAL59 and change in pH of the meat.

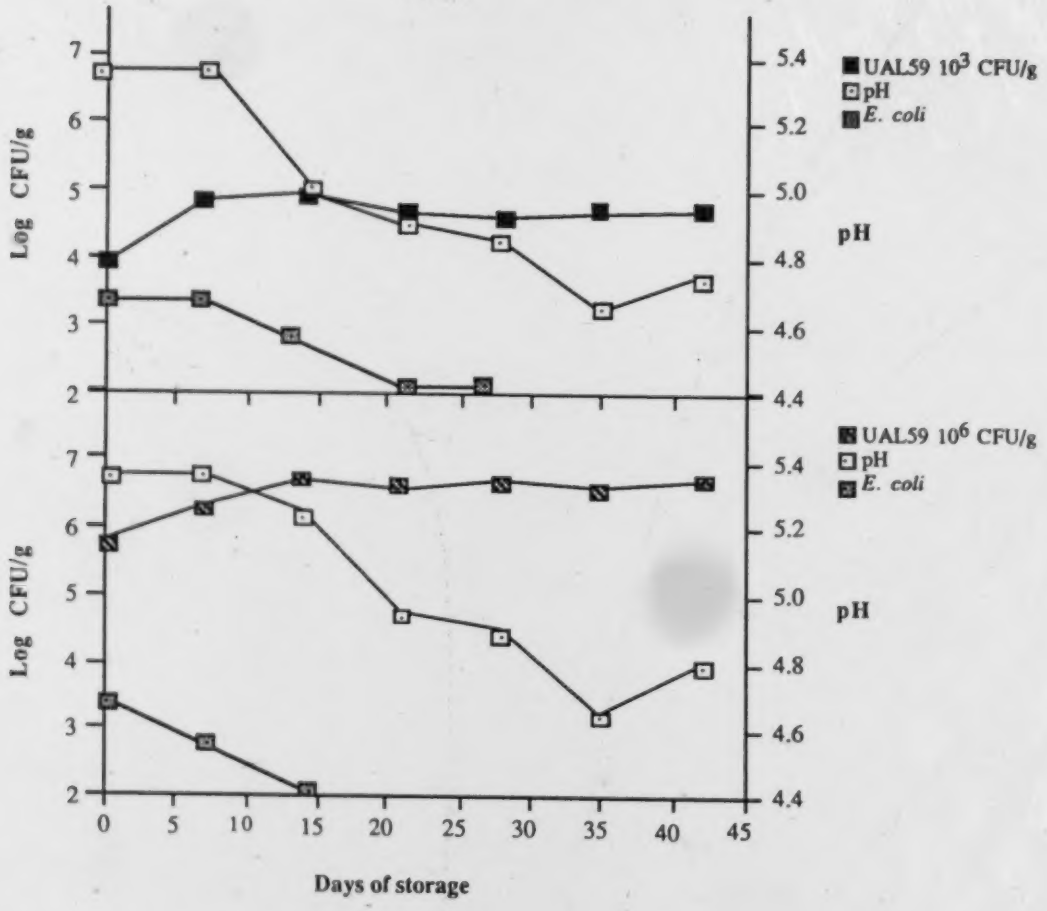


Figure 4.8. Survival of *Escherichia coli* at 1°C in the presence of LAB strain UAL59 and change in pH of the meat.

Table 4.3. Odor score for vacuum packaged ground beef stored at 10, 4, and 1°C over extended time.

Temp	10°C					4°C					1°C			
Strain	UAL3		UAL59			UAL3		UAL59			UAL3		UAL59	
CFU/g	10^3	10^6	10^3	10^6		10^3	10^6	10^3	10^6		10^3	10^6	10^3	10^6
Days					Days					Days				
2	N	+	N	+	4	+	N	N	N	7	N	N	N	N
4	+	+	+	+	7	+	+	N	+	14	2+	2+	2+	+
7	+	+	+	+	11	2+	2+	+	+	21	2+	2+	2+	2+
9	+	2+	+	+	14	2+	2+	+	+	28	3+	3+	3+	3+
11	2+	2+	+	+	18	2+	2+	2+	2+	35	3+	4+	3+	4+
14	2+	2+	+	3+	21	3+	3+	3+	3+	42	4+	4+	4+	4+
18	3+	3+	3+	4+	25	3+	3+	3+	3+					
21	4+	4+	4+	4+	28	3+	3+	3+	3+					
28	4+	4+	4+	4+	35	4+	4+	4+	4+					
					42	4+	4+	4+	4+					

N =normal smell
\+ =slight sour
2+ =sour
3+ =strong sour
4+ =decomposed or very sour

due to low pH. *K. pneumoniae* and *E. coli* increased in count by 100- and 1000-fold, respectively over the 5 day incubation period at 15°C, while the viable counts for *S. aureus* remained the same. *P. fluorescens* showed a 10-fold cycle decrease in number over this period.

$CaCO_3$ was more effective in buffering the meat pH above 6.0 compared with phosphate buffer. The starting pH of the meat after addition of the buffers was 6.5. After 5 days of storage at 15°C with added $CaCO_3$ the pH was at 6.1, whereas with phosphate buffer it had dropped to 5.5.

To check the inhibitory effect of low pH and lactate ions on the contaminating microflora without added LAB, ground beef was acidified with lactic or hydrochloric acid. Meat samples were γ-irradiated before inoculation to decrease the level and retard the growth of adventitious LAB. Samples were inoculated with the contaminating microflora at 10^5 CFU/g, vacuum packaged and stored at 4°C.

This study showed that *S. aureus, E. coli* and *K. pneumoniae* are not greatly affected by low pH during 10 days storage at 4°C. Only a 10-fold cycle decrease in the viable counts was observed. There was no noticeable difference between samples adjusted with lactic or hydrochloric acids. On the other hand, *P. fluorescens* was markedly affected by low pH and by lactate as illustrated in Figure 4.9. After 2 days of storage in meat adjusted to pH 4.5 with lactic acid, *P. fluorescens* counts dropped approximately 1000-fold and after 4 days of storage, *P. fluorescens* could not be isolated. In the presence of HCl (pH 4.5) *P. fluorescens* could still be detected after 10 days of storage.

4.1.7 Addition of catalase to inoculated ground beef

When catalase was added to ground beef inoculated with LAB strain UAL3 and contaminating microflora, no difference in viable count for the contaminating flora was seen compared with the control without catalase. This indicates that inhibition caused by

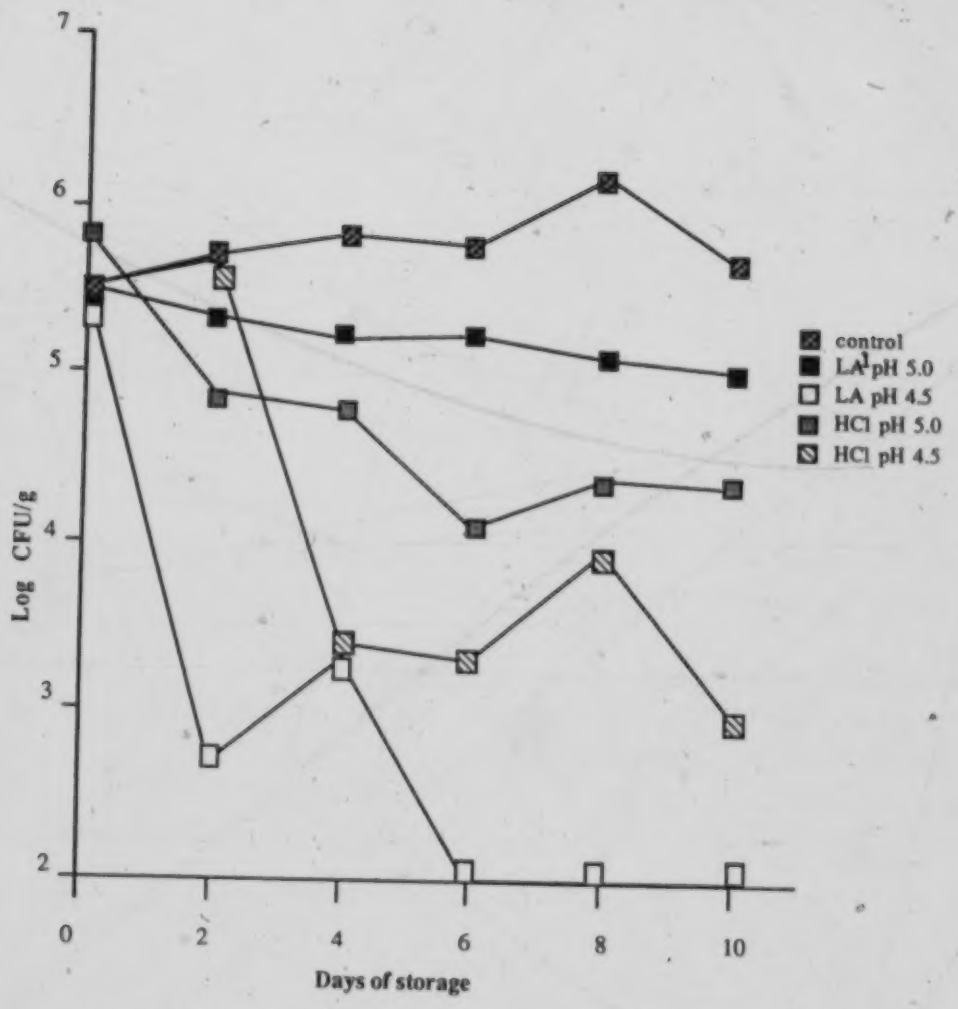


Figure 4.9. Effect of pH of ground beef on survival of *Pseudomonas fluorescens* in vacuum packaged ground beef at 4°C.

[1] LA = lactic acid

UAL3 is not due to the production of hydrogen peroxide. The pH of the ground beef with and without catalase was 6.6 after 48 h, whereas the unbuffered meat was at pH 4.9.

4.2 Factors influencing production of inhibitory substance by LAB

In an earlier study by Burns (1987) efforts were made to demonstrate the production of bacteriocin-like compounds in different broth and meat systems. Various methods of extraction and concentration of the inhibitory substances were tried, but the inhibitory effect of the bacteriocin-like compound could not be demonstrated. In this study a systematic approach to establish the production of inhibitory substance(s) by different LAB strains was undertaken.

4.2.1 Production of inhibitory substance on different growth media

Direct and deferred antagonism tests on different solid media showed that APT agar was the best culture medium for growth of the LAB producer and indicator strains. MRS at pH 5.6 and TSA gave poorer results for production of inhibitory substances. Autoinhibition was not observed when the producer strains were tested against themselves.

4.2.2 Production of inhibitory substance in solid and liquid media

Testing for production of inhibitory substances in the presence of the viable bacterial cells by direct and deferred antagonism compared with production in a liquid medium (extracellular production), showed greater inhibition by deferred than by direct antagonism. Comparison of these results with production of inhibitory substance in broth is shown in Table 4.4. In pH adjusted, (pH 6.5) heat treated (62°C for 30 min) supernatants, activity was only detected for strains UAL3, 4, 7, 8, 11, 72 and 86. This indicates that inhibition by deferred antagonism was in part or entirely due to acid production on the agar plate or that the inhibitory substance produced by some of these LAB strains is intracellular. No difference in result was observed between a regularly

Table 4.4. Inhibitory substance produced by selected strains of lactic acid bacteria on APT agar against a range of lactic acid bacteria indicator strains.[1]

Producer Strain	Indicator Strains														
	UAL3			UAL8			UAL9			UAL12			UAL15		
	D	I	S	D	I	S	D	I	S	D	I	S	D	I	S
UAL3	+	-	-	-	+	-	±	++	-	-	±	-	±	±	±
UAL4	-	±	-	-	++	++	+	++	++	-	-	-	-	-	-
UAL7	-	-	-	-	±	-	++	++	++	-	-	-	-	++	-
UAL8	-	++	+	-	±	-	++	++	++	±	++	-	-	-	-
UAL9	-	-	-	-	++	-	-	±	-	-	-	-	-	-	-
UAL11	±	++	++	+	++	++	++	++	-	-	±	-	+	++	±
UAL12	-	-	NT	±	+	NT	±	+	NT	-	±	NT	-	-	NT
UAL15	-	-	-	±	-	-	±	±	-	-	-	-	-	-	-
UAL16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UAL26	-	-	-	-	++	-	+	±	-	±	++	-	+	++	-
UAL59	-	++	±	±	++	±	±	++	-	-	±	-	-	+	-
UAL72	-	±	-	-	++	-	-	++	++	-	-	-	-	-	-
UAL86	-	±	-	-	++	+	-	++	++	-	-	-	-	±	-

[1] D = Direct antagonism.
I = Deferred antagonism.
S = Supernatant pH 6.5
NT = Not tested

++ = inhibition of indicator lawn with complete clearing and sharp edges.
+ = clearing with diffuse edges.
± = slight inhibition
- = no inhibition.

overlayered and an inverted overlayered plate indicating that the inhibitory activity was not due to the action of bacteriophage.

4.2.3 Production of inhibitory substance on APT agar

Results for the extraction of inhibitory substances produced on APT agar indicated that activity could only be recovered for strains UAL 8 and 11 against UAL9 as the indicator strain. The same result was observed for the regular and inverted overlayered agar procedure. Concentration of the extracts with the Amicon ultrafiltration apparatus, enhanced the inhibitory activity for UAL8 and UAL11 when tested against indicator UAL9. Better production of inhibitory substance occurred on APT with 0.5% agar than on regular APT agar, however differences in the extraction method might have influenced this result.

4.3 Production of inhibitory substance by strain UAL8 in culture medium and in ground beef

In the previous experiments, UAL8 showed strong inhibitory activity especially against UAL9 under different test conditions studied, and it was selected as a model strain for future meat inoculation experiments. Because UAL8 does not produce inhibitory substance at pH lower than 5.0 it was necessary to add a buffering system to keep the pH of the broth or meat at 6.0.

4.3.1 Extraction of inhibitory substance produced in heated and unheated ground beef

Results for inoculation of UAL8 into autoclaved and raw ground beef followed by heat treatment (62°C for 30 min) of the supernatants are shown in Table 4. 5. Inhibitory activity was detected in the raw and autoclaved ground beef with added distilled water, whereas no activity was detected in the raw meat without added water. This indicated either that binding of the inhibitor in meat occurred during heating or that the inhibitor was

Table 4.5. Production of inhibitory substance by strain UAL8 in raw and autoclaved ground beef and its heat stability (62°C for 30 min.).

	CFU/g	pH	Inhibition after heat treatment of extract
CMM	9.3×10^8	6.3	++[1]
BM broth	9.1×10^8	5.1[2]	++
Ground beef (autoclaved) + 10 mL distilled water	5.7×10^8	6.8	++
Ground beef (raw) + 10 mL distilled water	2.9×10^8	6.7	++
Ground beef (raw), no water added	6.6×10^8	6.4	-

[1] see footnote of Table 4.4.
[2] pH adjusted to 6.5 before testing inhibition.

not produced. However when the organism was grown in successive dilutions of meat, binding of the inhibitory substance did not occur.

4.3.2 Extraction of inhibitory substances from diluted ground beef

Dilutions of ground beef inoculated with a constant number of cells of strain UAL8 or with a standard amount of inhibitory substance from UAL8 showed similar results, as shown in Table 4.6. With increased dilution of the meat it was possible to recover the inhibitory substance after heat treatment. The inhibitory substance added to the meat showed less activity than when it was produced in the meat by UAL8. When the cells of UAL8 were added to dilutions of the meat, no decrease in activity was seen as a function of dilution of the meat . Millipore filter sterilization gave negative results for all dilutions, indicating a probable binding of the inhibitory substance to the filter membrane.

4.3.3 γ-Irradiation of inoculated ground beef to inactivate the producer strain UAL8

Ground beef inoculated with producer strain UAL8 and stored at 15°C for 48 h was γ-irradiated to inactivate the LAB strain. Loss of inhibitory activity was observed during the 22-h irradiation period compared with the untreated (unirradiated) control sample (Table 4.7). Inoculated ground beef treated with protease (1 mg/g) showed loss of inhibitory activity. In ground beef with addition of concentrated inhibitory substance, little inhibitory activity was detected after γ-irradiation. There was a complete loss of activity when the unconcentrated inhibitory substance was used.

In a similar experiment, meat samples were irradiated during extraction with buffer. This improved recovery of the inhibitory substance from the meat (Table 4.7). The concentrated inhibitory substance added to ground beef showed less activity than the sample inoculated with strain UAL8. Irradiation (7 kGy) of the inhibitory substance did not cause a reduction of activity. The sterility of the meat samples after irradiation was

Table 4.6. Activity of inhibitory substance produced by strain UAL8 in diluted ground beef.[1]

Ground beef dilutions	UAL8 inhibitory substance inoculated into dilutions of ground beef	Strain UAL8 (10^7 CFU/g) inoculated into ground beef and incubated at 15°C for 48 hours	
	Heated [2]	Heated	Filtered [3]
10 g	NT [4]	-	-
1 g + 0.5 mL [5]	-	-	-
1/2	-	+	-
1/4	±	++	-
1/8	++	++	-
1/16	+	++	-

1 see footnote Table 4.4
2 heated at 62°C for 30 min.
3 millipore filter, 0.45µm
4 NT = not tested
5 starting amount for twofold meat dilutions

Table 4.7. Effect of γ - irradiation of inoculated ground beef after storage at 15°C for 48 h on activity of inhibitory substance of strain UAL8 using two methods for extraction.

Sample/treatment	Inhibition of indicator UAL9
Extraction buffer added after irradiation	
Irradiated ground beef (7 kGy)	±
Protease-treated ground beef (1 mg/mL)	-
Inhibitory substance (concentrated) added to ground beef	±
Inhibitory substance (not concentrated) added to ground beef	-
Not irradiated ground beef (control)	++
Extraction buffer added before irradiation	
Vacuum packaged storage of ground beef	+
Modified atmosphere storage of ground beef	+
Inhibitory substance added (concentrated)	±
Inhibitory substance in BM broth (concentrated)	++

verified by inoculating 20 µL of treated supernatant into BM broth. Meat samples were VP or stored under modified atmosphere (10% CO_2 and 90% N_2). No difference in production of the inhibitory substance was observed. These results showed that extracting the inhibitory substance from ground beef with buffer during irradiation facilitated the extraction process.

An experiment was designed to determine whether a shorter extraction period in phosphate buffer could be used. When the samples were equilibrated for 2, 4 and 8 h in buffer, only slight or no inhibition was detected. In broth and meat with added inhibitory substance, good activity was detected after γ-irradiation for 20-22 h (7 kGy). The control sample showed greater inhibitory activity after 2 h than after 20 h in the extraction buffer. Bacterial counts of strain UAL8 were 2.7×10^7 CFU/g at time zero and 4.4×10^8 CFU/g after 48 h at 15°C.

4.3.4 Production of inhibitory substance by strain UAL8 in ground beef stored at 4°C

Production of inhibitory substance by strain UAL8 in CMM and BM broth could be detected after 3 days of storage at 4°C. Samples of ground beef with and without 1% $CaCO_3$ were inoculated with strain UAL8 at 1.8×10^7 CFU/g. Inhibitiory substance was detected in the extract from ground beef containing $CaCO_3$ compared with no activity in the extracts from unadjusted ground beef.

Addition of 1.9% filter sterilized glycerophosphate (disodium salt, Sigma) to buffer the meat initially above pH 6.0 resulted in less activity compared with 1% $CaCO_3$ (Table 4.8). Good inhibition was detected when strain UAL8 was grown in CMM or BM broth with added glycerophosphate.

Production of the inhibitory substance was detected in buffered meat after 4 days of storage at 4°C. After 8 days of storage, a sharp decline in activity occurred, and at 12 days inhibitory activity could not be detected (Figures 4.10 and 4.11). From an initial inoculum

Table 4.8. Effect of buffering system on production of inhibitory substance by strain UAL8 in ground beef at 4°C.

Sample treatment	pH after storage at 4°C		LAB counts after storage (CFU/g)		Inhibition	
	Days		Days		Days	
	5	10	5	10	5	10
Meat + UAL8 (10^7CFU/g)						
+ 1% $CaCO_3$	6.0	6.1	1.5×10^9	1.8×10^9	++[1]	+
+ SGP[2]	5.7	5.6	6.0×10^8	6.9×10^8	+	±
no added buffer	5.2	5.4	2.5×10^5	4.4×10^7	-	-
Meat, no added UAL8						
+ 1% $CaCO_3$	6.2	6.5	7.2×10^7	5.0×10^7	-	-
no added $CaCO_3$	5.3	5.5	2.4×10^5	1.6×10^7	-	-
Broth media						
CMM	6.0	6.0	NT	NT	++	++
CMM + SGP	6.4	6.4	NT	NT	++	++
BM + SGP	5.7	5.2	NT	NT	++	++

[1] see footnote of Table 4.4.
[2] Disodium glycerophosphate, 1.9%
[3] NT not tested

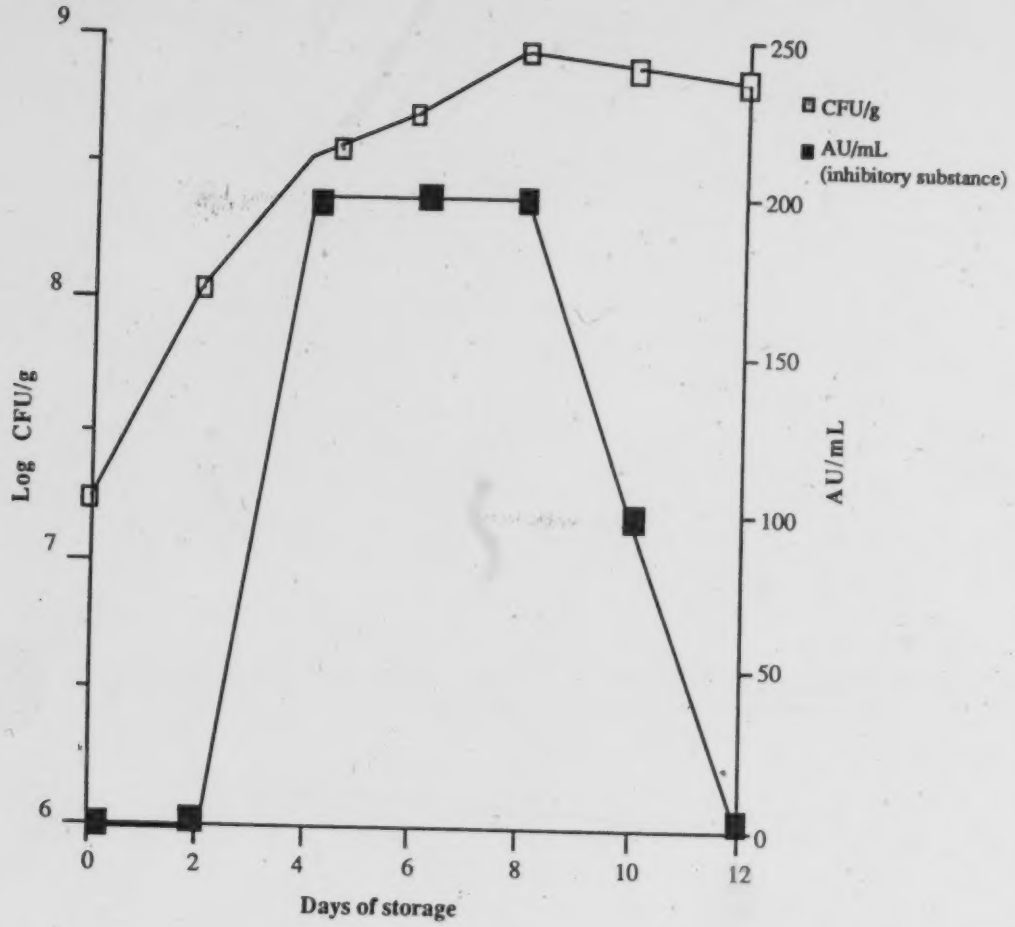


Figure 4.10. Growth of strain UAL8 in ground beef at 4°C and detection of inhibitory substance in the meat extract.

AU/mL = reciprocal of highest dilution showing inhibition of the indicator strain

Days of storage

Figure 4.11. Production of inhibitory substance by strain UAL8 in vacuum packaged ground beef stored at 4°C.

of strain UAL8 at 1.7 x 10^7 CFU/g a steady increase of viable cell numbers was noted up to 8 days, reaching 8.7 x 10^8 CFU/g.

4.3.5 Loss of activity of inhibitory substance in ground beef

In contrast to the stability of the inhibitory substance produced in broth culture to heat and membrane filtration, activity was lost in meat extracts by heat treatment (62°C for 30 min). Inhibitory substance added to meat and extracted after 2 and 24 h of storage at 1°C showed decreased activity after 24 h. Differences in activity were also detected as a result of the methods of sterilization of the meat extracts (Table 4.9). Inhibitory substance added to meat at 200 AU/g and immediately heat treated or filtersterilized, showed no inactivation by heat treatment, Millipore (0.45 µm) or Acrodisc (0.45 µm) filter sterilization. Within 2 hours of storage of the meat at 1°C, losses of activity were observed. After 24 h no activity could be recovered using Millipore membrane filtration of the meat extracts, whereas with Acrodisc filtration activity was retained. Filter sterilization of the extracts from ground beef (5 days at 4°C) showed similar results to those illustrated in Figure 4.12.

The inhibitory substance produced in ground beef by strain UAL8 at 4°C was extracted with buffer and filter sterilized through Acrodisc filters (0.45 µm). To check the nature of the inhibitory substance other characteristics such as sensitivity to protease (1 mg/mL) or inactivation by catalase (100 units/g) was tested. The results are summarized in Figure 4.12.

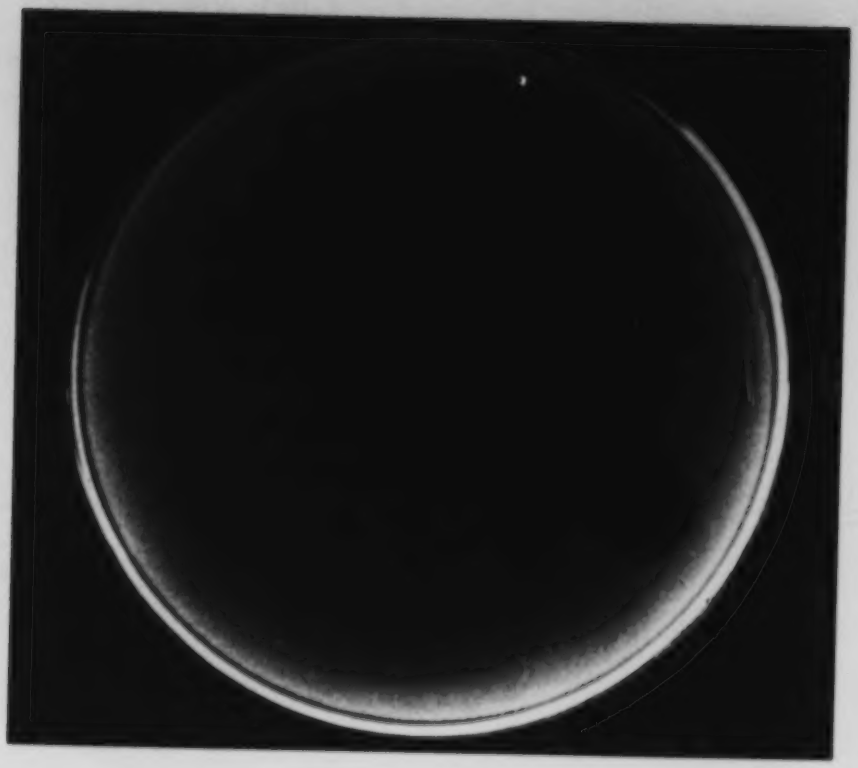
4.3.6 Activity of inhibitory substance added to ground beef and stored at 4°C

In previous experiments it was shown that activity in ground beef decreased faster during γ-irradiation at room temperature (20°C) and storage at 15°C compared with storage at 1°C. Strain UAL8 inhibitory substance was added to ground beef and stored at

Table 4.9. Inactivation of inhibitory substance added to ground beef using different methods for sterilization of cell supernatant.

Treatment	Time of storage at 1°C (h)		
	0	2	24
	Activity of inhibitory substance[1]		
Millipore filter (0.45 µm)	++	+	-
Heat (62°C for 30 min)	++	+	±
Acrodisc filter (0.45 µm)	++	+	+

[1] see footnote of Table 4.4



Treatments of supernatants and extracts

	A Protease 1 mg/mL	B Control no treatment	C Catalase 100 units/ml.
Meat	D Heat 62°C for 30 min	E Acrodisc filter 0.45µm	F Millipore filter 0.45µm
Broth	G Heat 62°C for 30 min	H Acrodisc filter 0.45µm	I Millipore filter 0.45µm

Figure 4.12. Nature of inhibitory substance produced by lactic acid bacterium UAL8.

4°C. During the first 24 h no loss in activity occurred (Figure 4.13), after 72 h of storage the activity dropped to 200 AU/mL, however, even after 5 days of storage some activity remained (100 AU/mL).

4.3.7 Effect of pH, glucose concentration and storage temperature on the production of inhibitory substance by strain UAL8 in broth

Both temperature and pH affect the production of inhibitory substance by strain UAL8. This is shown in Table 4.10 for the 2% glucose concentration. Similar results were observed with 0.2% glucose. At 25 and 10°C a similar increase in activity was seen with time of incubation. At 4 and 1°C activity increased at a much slower rate proportional to the slower growth rate. Growth of the test organism at the two carbohydrate concentrations gave similar results for production of inhibitory substance at all storage temperatures, with the exception of 4°C. At 4°C the inhibitory substance was only detected in BM broth containing 0.2% and not 2% carbohydrate. pH had a marked effect on production of the inhibitory substance. Good activity was detected in the controls (pH 6.6-6.7 in BM broth), and at pH 6.0. At pH 5.5 some activity was detected at 10°C for both carbohydrate concentrations. Only trace amounts of activity were detected at other temperatures. No activity was recorded at pH 5.0 at any storage temperature or glucose concentration.

During incubation, pH of the unadjusted controls dropped below 5.0 within 2 days at 25°C, 8 days at 10°C and 16 days at 4°C. In samples stored at 1°C the pH stayed above 5.0 for up to 20 days. The rate of drop in pH for broth adjusted to 6.0 or 5.5 was similar to the controls at both carbohydrate concentrations. In broth adjusted to pH 5.0, growth of strain UAL8 did not occur. Control samples of BM broth with added inhibitory substance (200 AU/mL), stored under different testing conditions, retained its activity at 200 AU/mL.

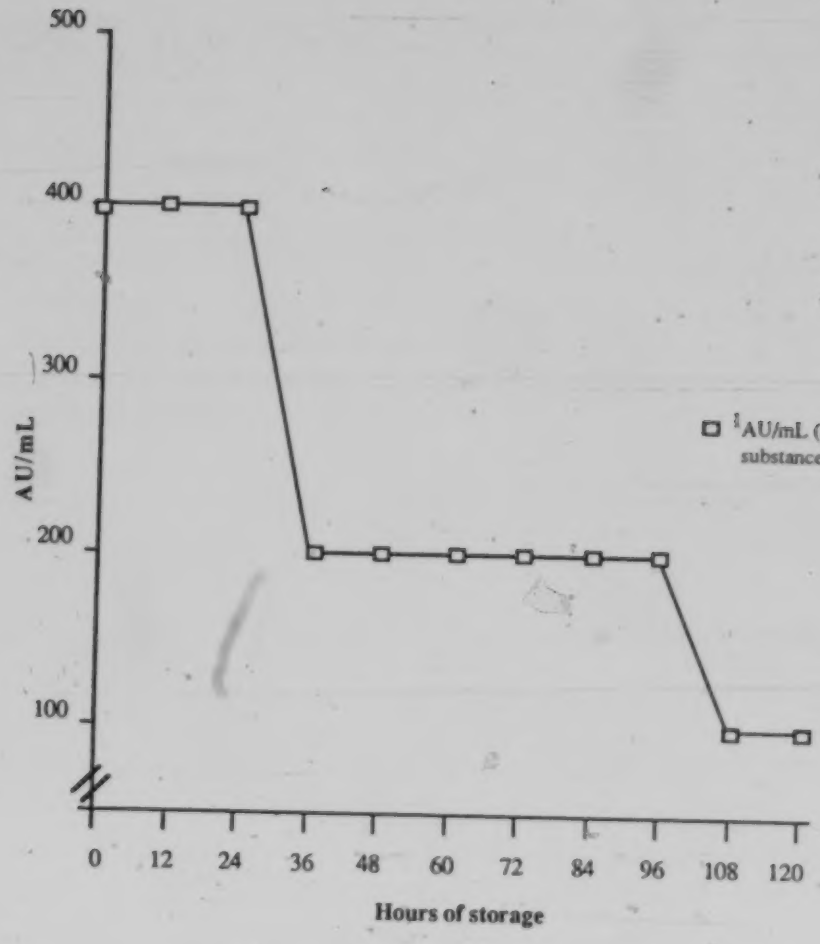


Figure 4.13. Loss of activity of inhibitory substance from strain UAL8 inoculated into ground beef stored at 4°C.

[1] AU/mL=arbitrary activity units of inhibitory substance/mL calculated from the reciprocal of the highest dilution showing inhibition of the indicator strain

Table 4.10. Growth and production of inhibitory substance by lactic acid bacterium UAL8 in BM broth with 2% glucose at pH 6.0 and 5.5 at different temperatures.

	25°C			10°C			4°C			1°C		
	time (days)	max. log CFU/mL	activity AU/mL	time (days)	max. log CFU/mL	activity AU/mL	time (days)	max. log CFU/mL	activity AU/mL	time (days)	max. log CFU/mL	activity AU/mL
pH 6.0	1	8.61	100	2	8.48	200	4	8.22	50	4	7.60	ND [1]
	2	8.80	100	4	8.67	200	8	8.81	100	8	8.60	50
	3	8.64	100	6	8.61	100	12	8.66	100	12	8.51	100
	4	8.68	100	8	8.67	100	16	8.70	100	16	8.45	50
	5	8.51	100	10	8.79	200	20	8.60	100	20	8.53	100
pH 5.5	1	8.34	<50	2	8.26	50	4	7.83	<50	4	7.34	ND
	2	8.32	ND	4	8.38	50	8	7.59	<50	8	7.95	ND
	3	8.40	<50	6	8.31	50	12	8.27	ND	12	8.12	ND
	4	8.35	ND	8	8.57	<50	16	8.58	ND	16	8.06	ND
	5	8.28	ND	10	8.55	<50	20	8.45	<50	20	8.03	ND

[1] ND = no detectable activity

Viable cell counts for strain UAL8 grown under the different test conditions showed that temperature, pH and carbohydrate concentration had a marked effect on growth. At the four incubation temperatures active growth occurred at both carbohydrate concentrations, with decrease in maximum cell population at lower pH levels. At 4 and 1°C a slower growth rate was observed at 0.2% carbohydrate concentration than at 2% (Figures 4.14 and 4.15). Control samples reached higher viable cell counts than the pH adjusted test samples at all four incubation temperatures.

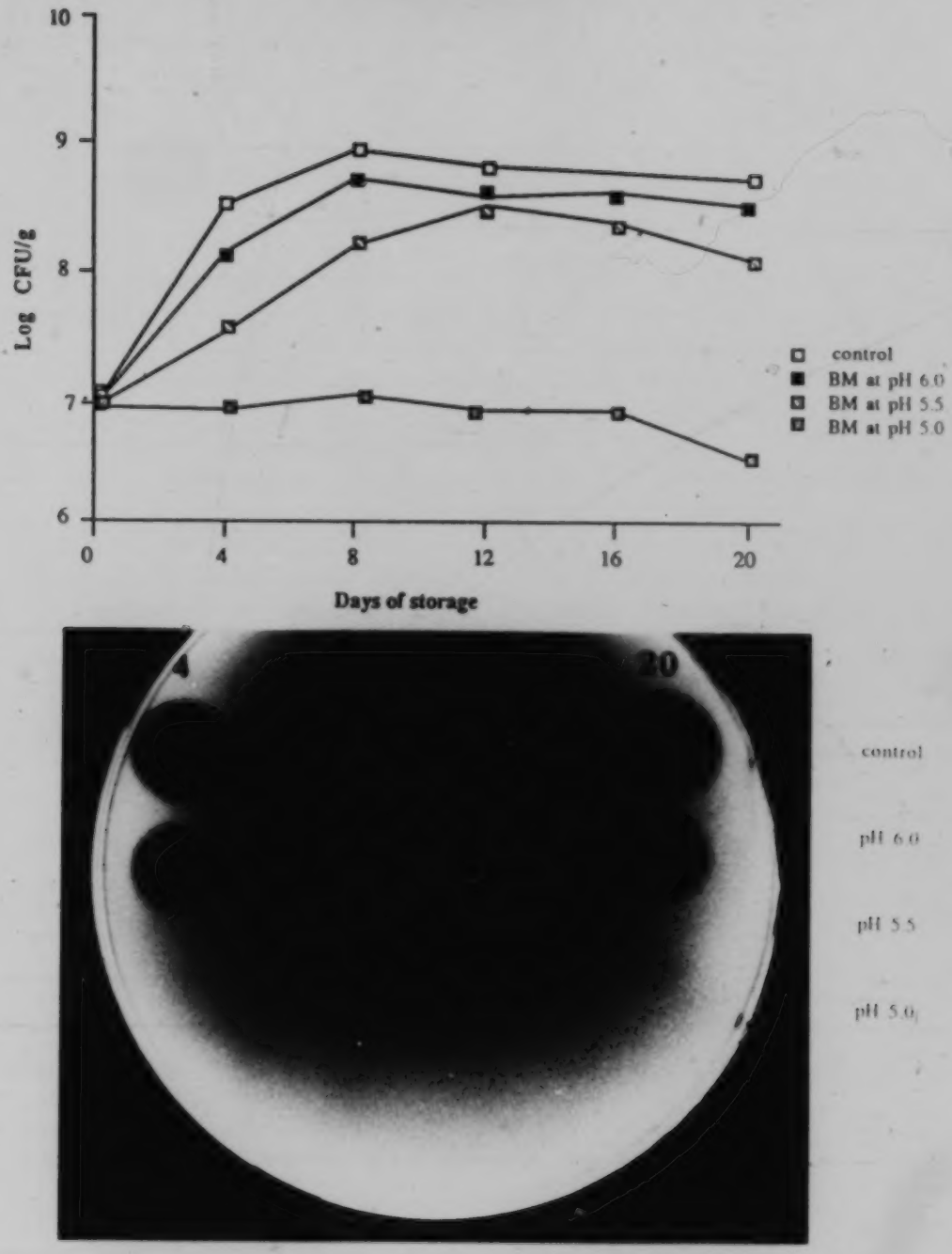
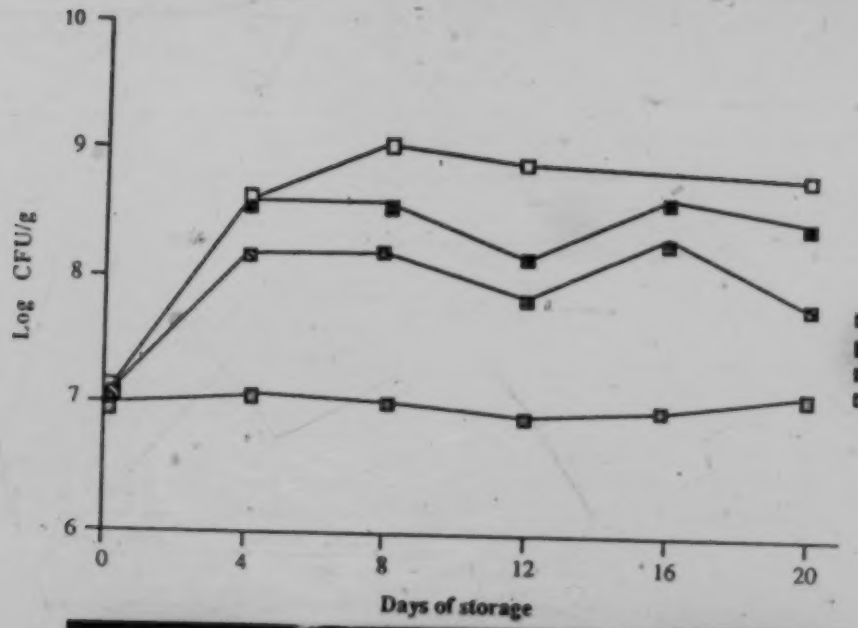


Figure 4.14. Growth of lactic acid bacterium UAL8 and production of inhibitory substance in BM broth containing 2.0% glucose at 4°C.



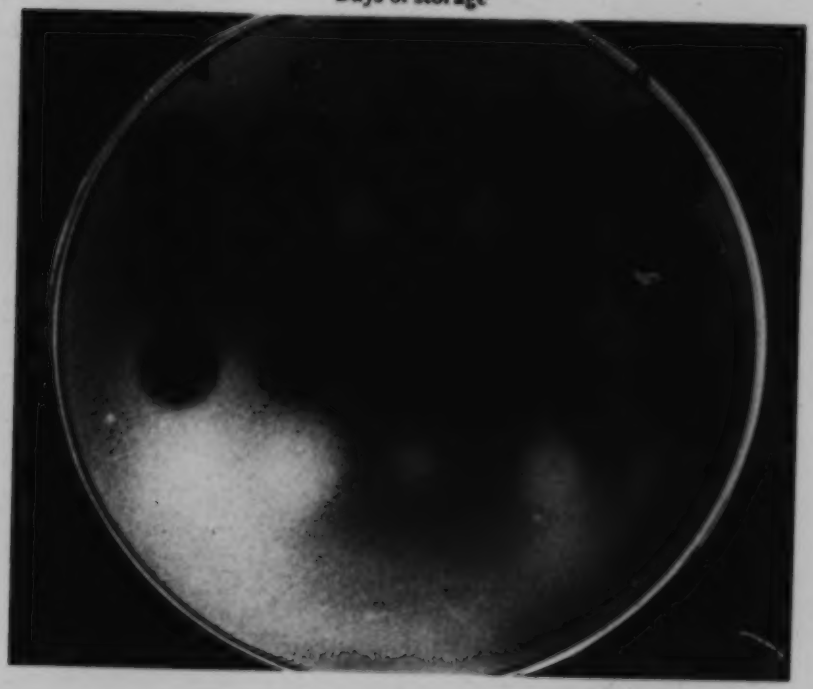


Figure 4.15. Growth of lactic acid bacterium UAL8 and production of inhibitory substance in BM broth containing 0.2% glucose at 4°C.

5.DISCUSSION

Lactic acid bacteria are able to grow under environmental conditions that occur in different foods, because they do not require O_2, they are resistant to CO_2 and are able to grow in relatively high salt concentrations (Egan, 1983). When meat is stored in an O_2 impermeable film, LAB rapidly become the predominant population, and outgrow the gram negative aerobic spoilage microflora. Meat packaged in low permeability film consumes O_2 during respiration and CO_2 is produced, resulting in an atmosphere of 20-40% CO_2 and less than 1% O_2. This constitutes a favorable environment for the growth of LAB. The ability of LAB to grow and reach high numbers in meat varies among strains of LAB, size of starting inoculum, storage temperature and gas atmosphere during storage (Christopher *et al*, 1979; Hanna *et al*., 1980). Earlier studies with LAB added to meats have shown that spoilage and pathogenic microflora can be inhibited (Reddy *et al*., 1970, 1975; Daly *et al*. 1972; Raccach *et al*. 1979). The strains used in these studies were usually of dairy origin and incubation was done under aerobic conditions.

In our study, LAB strains isolated from meats were used. Strains UAL3 and UAL59 were selected because of basic differences in their physiological characteristics. Strain UAL3 grows well at refrigerator temperatures and appears to be nonbacteriocinogenic. In contrast, UAL59 was selected for its inhibitory action resembling a bacteriocin active against a relatively wide range of indicator organisms, despite its poor growth at 1 and 4°C. It was anticipated that UAL59 would inhibit growth of spoilage and potentially pathogenic bacteria under conditions of temperature abuse, for example 10°C or higher.

UAL59 inoculated at low concentration into ground beef (10^3 CFU/g) grew poorly, reaching maximum population of 10^7 CFU/g at 10°C; while UAL3 with the same starting inoculum reached 10^8 CFU/g. UAL59 did not compete with the natural meat microflora when inoculated at 10^3 CFU/g. A large number of LAB colonies grew on the plates, probably corresponding to other LAB that do not develop well on MRS agar, as reported

by Egan (1983). These organisms were suppressed when UAL59 was inoculated in higher numbers, indicating an inhibitory effect of UAL59 on the adventitious meat microflora. The adventitious LAB in ground beef reached a maximum of 10^7 CFU/g after 14 days of storage. This is in agreement with work done on other VP meats such as beef steaks (Pierson *et al.*, 1970), lamb chops (Newton *et al.*, 1977), pork loins (Christopher *et al.*, 1980), and ground beef (Lee *et al.*, 1984), where the same maximum counts for adventitious LAB were found per cm^2 of meat surface (with the exception of ground beef study in which results expressed per gram of meat).

The growth of *B. thermosphacta* in VP meats is mainly controlled by 2 factors, pH of the meat and availability of O_2. Campbell *et al.* (1979) found that in VP beef at pH less than 6.0, the growth of *B. thermosphacta* depends on the availability of O_2 in the package. Above pH 6.0 the bacterium grows even in the presence of very low amounts of O_2, reaching concentrations of 10^7 CFU/cm^2. Roth and Clark (1975) suggested that the growth of *B. thermosphacta* was suppressed when LAB were present in high numbers in VP meats. Because CO_2 is not inhibitory to this bacterium, they suggested that an inhibitory compound produced by LAB may have caused the inhibition. They stated that it could not be an acidity effect because *B. thermosphacta* also produces acid. Collins-Thompson and Rodriguez Lopez (1980) also reported an inhibitory effect of LAB on the growth of *B. thermosphacta*, specifying that among a group of LAB, *Lactobacillus brevis* and *L. plantarum* showed the greatest antagonism toward *B. thermosphacta*. Contradictory results for inhibition of *B. thermosphacta* were observed in this study.

Our results indicate that inoculation of ground meat with 10^6 CFU/g of LAB inhibited the growth of the added pathogenic and spoilage microflora. One of the first studies in the use of LAB as a biologic system for the control of undesirable microflora in meats was done by Reddy *et al.* (1970), in which milk starter cultures were inoculated into aerobically stored ground beef. Daly *et al.* (1972) demonstrated inhibition of pathogenic and spoilage microflora in different foods by various LAB strains. The nature

of the inhibitory activity of LAB varies. Gilliland and Speck (1975) and Abdel-Bar and Harris (1984) reported that the inhibitory effect was due to the production of H_2O_2, whereas Sirvioe *et al.* (1977), Raccach and Henningsen (1984) and Tanaka *et al.* (1985) attributed the inhibition to an acidity effect. Park and Marth (1972) and Dubois *et al.* (1979) claimed that inhibition was caused by an inhibitory substance produced by LAB.

In this study the LAB strains showed different inhibitory activities against the added "contaminating" microflora. *S. aureus* was the least affected by the added LAB in the ground beef. *S. aureus* is resistant to adverse environmental conditions such as low pH, high salt concentration, low Aw and it can can grow under anaerobic conditions (Brock, 1974). According to King and Nagel (1967), *P. fluorescens* is inhibited at a CO_2 concentration of 10% or higher. In meats inoculated with LAB and stored under VP conditions, pH drops rapidly below 5.0 (approximately 11 days at 4°C) and CO_2 accumulates to around 20%. Both conditions may have contributed to the inhibition of *P. fluorescens* in this study. This strain was the most affected by the LAB added to the meat. *P. fluorescens* was only detected during a short period in the presence of LAB inoculated at 10^6 CFU/g compared with the control samples which had not been inoculated with LAB strains. *E. coli* and *K. pneumoniae* were also inhibited in the presence of the 10^6 CFU/g inoculum of LAB.

To rule out the possibility that the inhibitory effect was due to the production of H_2O_2, supernatants from LAB strains UAL3 and UAL 59 were treated with catalase. No difference in their inhibitory effect on a sensitive indicator strain was seen compared with the untreated control. When catalase was added to ground beef inoculated with UAL3, no difference in survival of the "contaminating" microflora was noticed compared with the ground beef without added catalase.

The pH of VP ground beef drops during storage due to accumulation of lactic acid produced by the LAB. The normal pH of fresh beef is 5.5. During anaerobic storage the pH drops to 4.5 - 4.6. The drop in pH was slower in the control samples in which the

adventitious LAB grew, than in the samples inoculated with LAB, even at the lower concentration of 10^3 CFU/g. The pH of the meat continued to drop after the LAB reached their maximum population of 10^7 to 10^8 CFU/g, indicating that acid production was independent from growth at maximum population.

Inoculum size of LAB did not influence the degree of souring. Grey discoloration of the meat occurred at all 3 storage temperatures and with both strains of LAB. More favorable color preservation was observed in the presence of UAL59 added at 10^6 CFU/g. The effect on meat color was probably more pronounced in this study because only small amounts of ground beef (11 g) were used. Larger amounts representing commercial quantities might give a different result.

Schillinger and Luecke (1986) found that meat pieces (200 g each) inoculated with LAB and stored for 30 days at 2°C only showed slight grey discoloration in the meat juice after the storage period had expired. Hanna *et al.*(1983) also found acceptable color and odor for VP beef steaks inoculated with LAB and stored for 28 days. Different results were found by Smith *et al.* (1980) when beef steaks were inoculated with LAB. Pronounced surface discoloration and high incidence of off odors were reported compared with uninoculated controls. Color and odor variations of meat inoculated with LAB remain a difficulty in the possible application of LAB as starter cultures in fresh meats. Differences in organoleptic characteristics reported between studies could depend on the strain of LAB, gas permeability of the wrapping or type of meat.

The addition of lactic and hydrochloric acids to VP ground beef with added "contaminating" microflora showed that at pH 4.5 there was a marked inhibitory effect of *P. fluorescens*. The inhibition was more pronounced for lactic than hydrochloric acid, especially at pH 4.5. *S. aureus*, *E. coli* and *K. pneumoniae* were not inhibited to the same extent by low pH or in the presence of lactate. Daly *et al.* (1972), in a similar study done in broth, observed no inhibition of *S. aureus* or *P. fluorescens* when these strains were grown in associated culture with *Streptococcus diacetilactis* in a medium controlled at pH

6.8 with sodium carbonate. They also observed that HCl allowed bacterial growth at all pH levels tested, while lactic acid showed a 100-fold decrease in bacterial counts at pH 4.5. They concluded that low pH could not have been the only inhibitory factor against *S. aureus*. In our study, *S. aureus* was more resistant to acid conditions which might have been due to differences in experimental conditions between studies (such as meat vs broth and absence vs presence of LAB). Nassos *et al.* (1985) stated that the lactic acid produced during VP storage extends shelflife of ground beef when subsequently exposed to O_2 in retail packages.

From the results of this study it appeared that strain UAL3 would cause souring resulting in rejection of the meat. In contrast UAL59 was selected for its apparently bacteriocinogenic quality. Production of bacteriocin-like compounds in meats should allow the producer strain to predominate the population. It was hoped that the special properties of UAL59, namely poor growth at normal refrigeration temperature below 5°C, but growth and production of inhibitory substance at abusive storage temperatures might be valuable attributes for extending the shelflife of VP ground beef. Unfortunately the results were equivocal. Further studies with UAL59 revealed that it no longer produced the inhibitory compound. As a result, studies on the growth of this strain in meats were not considered appropriate. Studies were designed that would emphasize the ability of LAB to produce inhibitory substances in meat and the persistence of these substances despite the presence of proteases in meat.

Attention was therefore focused on strain UAL8 that was shown to be a reliable producer of inhibitory substance(s) in broth culture. This strain is nonaciduric which; according to Shaw and Harding's (1984) classification, implies that the organism will not initiate growth at pH 3.9 and that after 1 week in La broth it produces a pH of approximately 4.7. Such an organism would be expected to initiate growth in fresh ground beef (pH approximately 5.5 to 5.6). Strain UAL8 was shown to be a prodigious producer of inhibitory substance(s), in that inhibition of a susceptible indicator strain can be detected

early in the logarithmic phase of growth (Stiles *et al.* 1987, unpublished data). Because not much information is available in the literature showing the presence of bacteriocin-like compounds in meats, studies on the use of LAB as inhibitory substance producer strains for use as potential starter organisms in meat would be done. To determine the production of inhibitory substances produced by LAB isolated from meat, screening procedures such as direct and deferred antagonism and production of the inhibitory substance in broth were done. Direct and deferred antagonism do not differentiate inhibition due to acid production, often giving false positive results. In direct antagonism testing there is no time for diffusion of the inhibitory substance into the growth medium. With direct antagonism the release of the inhibitor is tested during early phase of growth (Tagg *et al.*, 1976). Most of our bacteriocinogenic meat isolates were shown to be positive by the deferred antagonism test.

Bacteriocin production can be cell associated or extracellular. When inhibitory substances are secreted into the growth medium, they can be recovered from the supernatant and tested. According to Tagg *et al.* (1976), the proportion in which cell bound and extracellular forms of bacteriocins are present could depend on the composition of the growth medium. *S. aureus* 414 bacteriocins is bound to the cell surface and can only be released by mechanical disruption of the cells (Gagliano and Hindsdill,1970), whereas Helveticin J produced by *Lactobacillus helveticus* is recovered from culture supernatant (Joerger and Klaenhammer, 1986). In this study, activity was detected in broth supernatants indicating that these cultures produce extracellular inhibitory substances.

Detection of inhibitory substances was also tested on solid media in an effort to mimic the production in a meat system. Only 2 of the 11 strains produced inhibitory substances under these conditions. Bacteriocin production could be favored by growth on semisolid culture media, such as that used by Barefoot and Klaenhammer (1983). Results indicated that strain UAL26 produced its inhibitory substance under this condition, but it could only be detected after concentration of the extract. Poor recovery on solid media

differs from the report of Barefoot and Klaenhammer (1983) in which a variety of strains was shown to produce inhibitory substances by this procedure.

Inhibitory substances such as bacteriocins bind nonspecifically to different surfaces (Upreti and Hindsdill, 1975) and such binding may occur when they are produced in meats. This phenomenon may explain the failure to detect activity during extraction from ground beef, as opposed to the positive result when water was added to meat system. Since the inhibitory substance apparently binds with the meat proteins, when the extract is heated and protein precipitation occurs it is assumed that the inhibitory substance is coprecipitated. However dilutions of the meat showed recovery of activity indicating that as more water was present less binding of the inhibitory substance to the meat occurred.

To determine the presence of inhibitory substance in meat the LAB strain(s) must be inactivated to avoid direct antagonism by the viable cells. Since heating could not be applied because of the coprecipitation of the meat proteins and inhibitory substance, γ-irradiation of the ground beef was used as an alternative. Lactobacilli are more resistant to γ-irradiation than other bacteria (Hastings *et al.*, 1986) and therefore a dose of 7 kGy had to be applied which required a long period of exposure (20-22 h). This resulted in the inhibitory substance produced in the ground beef being exposed to proteolytic meat enzymes at room temperature for that period of time. This resulted in poor recovery of inhibitory substance and made it necessary to find a method of keeping the samples at low temperature during irradiation. It was shown that addition of buffer to the ground beef during irradiation helped in the extraction process, but that the activity of inhibitory substance added to the meat was also lost during irradiation. To slow down the proteolytic activity of meat enzymes during irradiation the samples were frozen. This, plus the added buffer, markedly improved extraction of the inhibitory substance from ground beef.

It was considered important to demonstrate that the inhibitory substance was produced in ground beef at 4°C. This has a practical application if LAB are to be used as starter cultures, because they should be able to produce their bacteriocin-like substances at

the storage temperatures of the VP meat. Bacteriocin production by LAB can occur during the log or stationary phases of growth, depending on the bacterial strain (Tagg *et al.*, 1976; Barefoot and Klaenhammer, 1984). According to results from our laboratory it was determined that strain UAL8 produces its inhibitory substance in the early log phase. The meat inoculation/extraction experiments showed that inhibitory activity from the meat extract was detected after 4 days of storage at 4°C, showing maximum activity at that time and maintenance of the activity up to 8 days. A sharp decline occurred thereafter and, at 12 days of storage inhibition could no longer be detected. This probably indicates that the bacterial population reached stationary phase, that the inhibitory substances are no longer being produced, and the rapid loss of activity may indicate activity of proteolytic enzymes. A similar activity curve was observed for Helveticin J by Joosten and Klaenhammer (1986), in which a stable level of activity was detected for a certain period followed by sharp drop in activity. Their study was done in an automatic controlled fermenter, where proteolytic enzymes could not interfere. No explanation was given for their result. This finding indicates that after 12 days of storage at 4°C, bacteriocin-like substances may no longer be able to inhibit undesirable flora in VP meats.

The addition of the concentrated inhibitory substance produced by strain UAL8 to ground beef stored at 4°C showed a period of several days where the activity remained the same. This experiment was done to demonstrate the influence of proteolytic enzymes from meat on inhibitory substance produced by strain UAL8. It seems likely that protease activity occurs, but at low temperature the inactivation of the inhibitory substance is slowed down.

Killing of the LAB cells by γ-irradiation was a cumbersome procedure, whereas the use of "Acrodisc" low protein binding filters to sterilize the meat extracts represented a marked improvement in the extraction procedure. It was important to extract the inhibitory substance from meat for 20-22 h to obtain good yields.

According to Tagg *et al.* (1976), bacteriocin production is usually greater at the optimum growth temperature of the LAB. This was also observed in our study where a decrease in activity occurred as the storage temperature dropped. The amount of glucose in meat is variable, but approximately 0.2% is accepted as an average amount (Newton and Gill, 1978; Greaser, 1986). The presence of carbohydrate can be important for the production of the inhibitory substance as discussed by Tagg *et al.*, (1976). The pH also plays an important role in production of inhibitory substance. Some microorganisms produce their inhibitory substances at low pH, for example Las 5 is produced around pH 5.0 (Zajdel *et al.*, 1985) whereas others are produced at higher pH of 6.5 to 8.0 (Jetten *et al.*, 1972). According to our results, glucose concentration did not have a marked influence on the production of inhibitory substance by strain UAL 8 in broth. In contrast pH had a noticeable effect, with good activity produced above pH 5.5.

This study confirmed that an added "contaminating" microflora in VP ground beef can be markedly affected by a LAB microflora. However, it became apparent that the role of inhibitory substance(s) produced by LAB isolated from meats requires further study and classification. It was demonstrated that the LAB strain UAL8 could produce its inhibitory substance(s) in VP ground beef, and that they remained active in meat stored at 4°C or below. Unfortunately, strain UAL8 is not ideal for use in a meat system, because it does not produce its inhibitory substance(s) at pH 5.5. Several options for further studies are available. It may be possible to select other LAB strains that produce their inhibitory substance(s) at lower pH. In on-going studies in the laboratory it has been shown that production of inhibitory substance(s) by strain UAL8 is most probably plasmid mediated. As a result, it may be possible to transfer production of inhibitory substance(s) to strains capable of growth and production of inhibitors at the pH of normal ground beef.

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